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THE BIOCHEMICAL JOURNAL

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The Effect of Insulin on Incorporation of Amino Acids into Protein of Normal Rat Diaphragm *in vitro*

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(*Received* 31 *January* 1958)

Sinex, MacMullen & Hastings (1952) found that, when insulin was added *in vitro* to diaphragm from normal non-fasting rats incubated in a medium containing DL-[1-^{14}C]alanine but lacking an easily oxidizable substrate such as glucose or pyruvate, the transfer of ^{14}C from the alanine into protein in the diaphragm was increased. Similar observations with [1-^{14}C]glycine in place of alanine were made by Krahl (1953). Sinex *et al.* (1952) found that transfer was less if glucose or pyruvate was present in the medium when no insulin had been added, and that in the presence of glucose or pyruvate addition of insulin raised to only a small extent incorporation into diaphragm protein of ^{14}C from the alanine. But Krahl (1953), on the other hand, observed that incorporation of ^{14}C from [1-^{14}C]glycine into protein of normal fasting rat diaphragm *in vitro* was raised by the addition of glucose alone to the medium, the presence of insulin in the medium in addition to glucose having no significant effect. Since these results were of importance with respect to the relative effects of insulin on carbohydrate and protein metabolism (see Ketterer, Randle & Young, 1957), we have repeated the experiments of these authors and extended their observations to include a variety of labelled amino acids.

MATERIALS AND METHODS

Radioactive materials. ^{14}C-Labelled materials were obtained from the Radiochemical Centre, Amersham. Glycine and DL-alanine, both 1-^{14}C-labelled, and uniformly ^{14}C-labelled ([U-^{14}C]) L-alanine were diluted to a specific activity of about 65 μc/m-mole. L-Leucine, L-isoleucine, L-phenylalanine, L-serine, L-lysine, L-arginine, L-glutamic acid and L-aspartic acid, all U-^{14}C-labelled, were diluted to a specific activity of 130 μc/m-mole. A radioactive protein hydrolysate, obtained by the acid hydrolysis of protein from *Chlorella* grown in the presence of $^{14}CO_2$ and containing a large number of [^{14}C]amino acids, was diluted with a mixture of amino acids (made by acid hydrolysis of *Chlorella*) to a specific activity of 130 μc/m-mole.

L-[^{35}S]Methionine, in the form of methionine sulphoxide, had a specific activity of 2·7 mc/mg. and was used at a concentration of 1 μc/flask.

Medium. Bicarbonate buffer (pH 7·4; Gey & Gey, 1936), gassed with $O_2 + CO_2$ (95:5, v/v), was used throughout. Amino acids had a final concentration of 2·7 μmoles/ml., except for methionine (0·2 μg./ml.). Glucose and sodium pyruvate were used at a concentration of 12·5 μmoles/ml., and NaCl was added to all media not containing sodium pyruvate in such amount as to keep the concentration of Na^+ ions of the medium constant.

Insulin (Boots crystalline insulin) was dissolved in 0·0017N-HCl to give a concentration of 10 units/ml. Insulin solution (0·1 ml., or 0·1 ml. of 0·0017N-HCl as control) was added to each flask to make a final insulin concentration of about 0·5 unit/ml.

Preparation of diaphragm. A female albino Wistar rat, which weighed 100–150 g. and had fasted 20–24 hr. before use, was decapitated and bled, and the diaphragm removed. The two halves of the diaphragm were washed in freshly gassed buffer, then gently blotted and transferred to small conical flasks containing 2 ml. of medium. One half of a diaphragm was used as control for the other half in experiments where the effect of insulin was studied. Two hemidiaphragms were added to each flask. The flasks were gassed, sealed and incubated at 37° in a Dubnoff shaker.

Preparation of protein from diaphragm. After incubation for 2 hr., the diaphragm was removed, blotted and homogenized in a Potter–Elvehjem homogenizer in 10 ml. of 10% trichloroacetic acid (TCA). The precipitate was spun down, resuspended in 10 ml. of 10% TCA and heated for 15 min. at 90°. The insoluble material was extracted twice with 1·5 ml. of 0·4N-NaOH, and the insoluble residue rejected (protein content about 5%). The dissolved protein was reprecipitated by the addition of 5 ml. of 10% TCA. It was washed with 2 ml. of ethanol–ether (50:50, v/v), then with 2 ml. of ether, and was dried in a vacuum desiccator.

In experiments with methionine, the TCA precipitate was heated for 15 min. at 90° and then dissolved in 1 ml. of 98% formic acid, followed by 0·2 ml. of 100 vol. hydrogen peroxide. The solution was allowed to stand for 30 min. at room temperature, and was then precipitated by the addition of 10 vol. of 10% TCA. The precipitate was washed with ethanol and ether and dried as above.

Assessment of radioactivity of diaphragm protein. Two hemidiaphragms, weighing about 200 mg., yielded by the method described 25–30 mg. of protein, which had a nitrogen content of about 13% (consistent with a protein content of about 80%), and about 0·05% of phosphorus. The samples of protein were ground in a mortar, collected on 1 cm.^{2} plastic disks, and counted as infinitely thick samples (Popják, 1950), by using a Geiger–Müller tube with a thin mica end-window (thickness 1·5–2·5 mg./cm.^{2}). Sufficient counts were recorded to give a counting error of less than 5%.

Statistical treatment of results. Experiments were performed in groups of six, one each with and without addition of insulin in the absence of added substrate, with and

without addition of insulin in the presence of glucose, and with and without addition of insulin in the presence of pyruvate. Counts found were expressed as a percentage of the counts of the control. This procedure was repeated in most cases six times. The percentages so obtained were grouped and averaged, and the standard errors determined. Significance was assessed on the basis of Student's t test.

RESULTS

Alanine. Our results with DL-[1-^{14}C]alanine agree with the findings of Sinex *et al.* (1952), since incorporation of ^{14}C from this amino acid into the protein of diaphragm, incubated in a medium free from easily oxidizable substrate, rose in the presence of insulin (Table 2). Likewise, we agree with them that with DL-[1-^{14}C]alanine addition of glucose or pyruvate to the medium depresses the incorporation into protein of the ^{14}C from the alanine, and that the effect of insulin in promoting incorporation of the ^{14}C under these conditions is less than is found in the absence of added glucose or pyruvate (Tables 1–3). Generally similar results were obtained when L-[U-^{14}C]alanine was used instead of DL-alanine (Tables 1–3), although the incorporation with L-alanine was significantly greater than that with DL-alanine.

Other [^{14}C]*amino acids.* When experiments similar to those with [^{14}C]alanine were carried out either with [1-^{14}C]glycine or with various L-[U-^{14}C]-amino acids added singly, or with algal [^{14}C]protein hydrolysate, incorporation of ^{14}C into the protein of diaphragm was unaffected by addition of glucose or pyruvate either in the absence or in the presence of insulin (Tables 1 and 3). The addition of insulin to the medium resulted in a greater incorporation of ^{14}C either in the presence or absence of glucose or pyruvate (Table 2).

With L-[U-^{14}C]leucine, and to a smaller extent with L-[U-^{14}C]glutamic acid, incorporation of ^{14}C into protein was slightly greater in the presence of added glucose than in the absence of added substrate, insulin also being absent. Addition of insulin in the presence of glucose raised further the incorporation of ^{14}C, and the increase produced by the addition of insulin was roughly the same in the presence of glucose as in its absence. Addition of pyruvate diminished slightly incorporation of ^{14}C from L-[U-^{14}C]glutamic acid into protein (Tables 1–3).

Incorporation into protein of ^{14}C from L-[U-^{14}C]-aspartic acid was unaffected by addition of glucose but was significantly depressed when pyruvate was

Table 1. *Effect of the addition of glucose or of pyruvate to the medium on the incorporation* in vitro *of radioactivity from amino acids into protein of normal rat diaphragm*

No insulin was added to the medium. The results are expressed as a percentage of the control value found in the absence of added glucose or pyruvate. The value of P for a difference which is significant is given in brackets. The number of observations is given in parentheses.

Labelled amino acid added to medium	Radioactivity in diaphragm protein: Control experiments (counts/min./disk)	Percentage of control value in presence of added: Glucose	Pyruvate
DL-[1-^{14}C]Alanine	57	62·6±3·0 (8) [P<0·001]	43·9±2·6 (8) [P<0·001]
L-[U-^{14}C]Alanine	103	65·5±3·2 (6) [P<0·001]	47·0±3·1 (6) [P<0·001]
[1-^{14}C]Glycine	84	108·5±5·3 (8)	88·0±9·6 (8)
L-[U-^{14}C]Leucine	524	115·0±6·6 (8) [P<0·1]	94·5±6·9 (8)
L-[U-^{14}C]Isoleucine	337	118·4±15·5 (6)	100·2±10·4 (6)
L-[U-^{14}C]Phenylalanine	104	102·7±5·4 (6)	100·7±5·6 (6)
L-[U-^{14}C]Serine	226	109·0±5·0 (6)	97·3±5·1 (6)
L-[U-^{14}C]Lysine	92	104·0±4·6 (6)	95·2±5·7 (6)
L-[U-^{14}C]Arginine	144	100·3±5·2 (6)	95·3±6·0 (6)
L-[U-^{14}C]Glutamic acid	34	110·0±3·4 (6) [P<0·05]	89·7±3·1 (6) [P=0·02]
L-[U-^{14}C]Aspartic acid	37	90·8±11·0 (6)	75·0±4·6 (6) [P<0·01]
L-[^{35}S]Methionine	382	130·8±9·4 (6) [P<0·05]	112·5±6·7 (6)
Algal ^{14}C-labelled protein hydrolysate	1800	101·0±4·8 (6)	93·7±3·4 (6)

Table 2. *Effect of the addition of insulin (0·5 unit/ml.) to the medium on the incorporation* in vitro *of radioactivity from amino acids into protein of normal rat diaphragm*

The results are expressed as a percentage of the control value found in the absence of added insulin. The value of P for a difference which is significant is given in brackets. The number of observations is given in parentheses.

	Medium including					
	No added substrate Radioactivity in diaphragm protein		Glucose Radioactivity in diaphragm protein		Pyruvate Radioactivity in diaphragm protein	
Labelled amino acid added to medium	Control experiments (counts/min./disk)	Percentage of control value in presence of added insulin	Control experiments (counts/min./disk)	Percentage of control value in presence of added insulin	Control experiments (counts/min./disk)	Percentage of control value in presence of added insulin
DL-[1-^{14}C]Alanine	57	135·7±4·8 (16) [P<0·001]	35	104·2±3·2 (8)	26	121·7±5·2 (8) [P<0·01]
L-[U-^{14}C]Alanine	106	129·0±6·0 (6) [P<0·01]	69	105·0±1·1 (6) [P=0·01]	49	135·7±3·7 (6) [P<0·001]
[1-^{14}C]Glycine	84	145·0±8·0 (8) [P=0·001]	91	141·0±7·3 (8) [P=0·001]	70	162·0±6·6 (8) [P<0·001]
L-[U-^{14}C]Leucine	524	117·9±1·8 (8) [P<0·001]	596	116·0±2·5 (8) [P<0·001]	491	121·7±2·9 (8) [P<0·001]
L-[U-^{14}C]Isoleucine	337	146·7±2·3 (6) [P<0·001]	422	156·5±5·9 (6) [P<0·001]	335	165·5±6·5 (6) [P<0·001]
L-[U-^{14}C]Phenylalanine	103	136·8±5·8 (6) [P<0·01]	106	138·7±3·2 (6) [P<0·001]	103	145·5±5·1 (6) [P<0·001]
L-[U-^{14}C]Serine	226	157·3±6·1 (6) [P<0·001]	244	140·2±4·1 (6) [P<0·001]	221	146·0±8·1 (6) [P<0·01]
L-[U-^{14}C]Lysine	92	161·8±9·2 (6) [P<0·01]	96	158·2±5·2 (6) [P<0·001]	89	172·7±5·2 (6) [P<0·001]
L-[U-^{14}C]Arginine	144	144·8±7·7 (6) [P<0·01]	144	139·0±7·6 (6) [P<0·01]	136	148·8±4·0 (6) [P<0·001]
L-[U-^{14}C]Glutamic acid	34	131·7±2·8 (6) [P<0·001]	38	125·7±6·0 (6) [P<0·01]	31	128·5±6·7 (6) [P<0·01]
L-[U-^{14}C]Aspartic acid	37	137·7±4·0 (6) [P<0·001]	33	124·0±3·4 (6) [P<0·001]	27	131·3±3·6 (6) [P<0·001]
L-[^{35}S]Methionine	382	217·8±7·0 (6) [P<0·001]	489	171·0±6·4 (6) [P<0·001]	426	216·0±10·2 (6) [P<0·001]
Algal ^{14}C-labelled protein hydrolysate	1800	154·8±4·3 (6) [P<0·001]	1810	152·5±6·2 (6) [P<0·001]	1690	154·7±6·2 (6) [P<0·001]

added to the medium. Addition of insulin, however, stimulated incorporation both in the presence and absence of added substrate (Tables 1–3).

With L-[^{35}S]methionine, the incorporation of ^{35}S into diaphragm protein was promoted by addition of insulin to the medium whether or not glucose or pyruvate were also present (Tables 1–3).

In these experiments the percentage rise in incorporation of label differed from one amino acid to another, but the results were of the same general order.

The results are summarized diagrammatically in Fig. 1.

DISCUSSION

Methods. In their experiments with alanine Sinex *et al.* (1952) used non-fasting male rats, whereas we have used fasting females. Nevertheless, we agree with their findings with respect to the stimulating action of insulin on the incorporation of ^{14}C from DL-[1-^{14}C]alanine into diaphragm protein, and the depressing effect on this incorporation of the presence of glucose or pyruvate in the medium. This observation is of particular interest, because the results of Krahl (1953) suggested that diaphragm from fasting animals can behave differently from diaphragm from non-fasting rats. In a few experiments carried out on the possible influence of sex we have found no differences in behaviour between diaphragm from male or female albino rats.

Behaviour of alanine. Although addition to the medium of glucose or pyruvate diminishes markedly incorporation of radioactivity from [^{14}C]alanine into protein of diaphragm, incorporation of label from none of the other labelled amino acids we have studied is so strikingly depressed by addition to the medium of glucose or pyruvate. We therefore are forced to conclude that the behaviour of alanine is atypical, and must be due to factors peculiar to alanine. Sinex *et al.* (1952) considered the possibility that the diminution in incorporation of radioactivity from [^{14}C]alanine in the presence of glucose or pyruvate results from a dilution of the labelled alanine with unlabelled alanine produced by a process of transamination (Braunstein & Kritzmann, 1937). But if this explanation were true, one would expect to find that addition of glucose or pyruvate to the medium would diminish incorporation of radioactivity from [^{14}C]glutamic acid, aspartic acid and possibly other amino acids. We have observed no such phenomenon in our experiments, except possibly in the effect of the addition of pyruvate on incorporation of radioactivity from [^{14}C]aspartic acid. Alanine differs from all the amino acids we have examined for reasons which are at present under investigation (Manchester & Young, 1958). If we assume that

Table 3. *Effect of the addition of glucose or of pyruvate to an insulin-containing medium on the incorporation* in vitro *of radioactivity from amino acids into protein of normal rat diaphragm*

Insulin (0·5 unit/ml.) was present in each experiment. The results are expressed as a percentage of the control value found in the absence of added glucose or pyruvate. The value of P for a difference which is significant is given in brackets. The number of observations is given in parentheses.

Labelled amino acid added to medium	Radioactivity in diaphragm protein: Control experiments (counts/min./disk)	Percentage of control value in presence of added: Glucose	Pyruvate
DL-[1-^{14}C]Alanine	73	49·8±2·7 (8) [P<0·001]	38·3±3·6 (8) [P<0·001]
L-[U-^{14}C]Alanine	130	58·8±3·1 (6) [P<0·001]	51·7±2·7 (6) [P<0·001]
[1-^{14}C]Glycine	119	105·4±2·4 (8)	97·6±9·3 (8)
L-[U-^{14}C]Leucine	622	112·5±4·5 (8) [P<0·05]	96·0±3·3 (8)
L-[U-^{14}C]Isoleucine	495	124·5±13·8 (6)	113·0±11·8 (6)
L-[U-^{14}C]Phenylalanine	141	104·2±4·3 (6)	105·5±2·7 (6)
L-[U-^{14}C]Serine	351	97·2±2·2 (6)	90·2±5·2 (6)
L-[U-^{14}C]Lysine	152	102·0±4·1 (6)	102·5±6·1 (6)
L-[U-^{14}C]Arginine	207	96·5±6·4 (6)	98·5±6·9 (6)
L-[U-^{14}C]Glutamic acid	45	104·7±3·8 (6)	88·2±6·7 (6)
L-[U-^{14}C]Aspartic acid	51	81·5±7·7 (6)	72·3±4·5 (6) [P<0·01]
L-[^{35}S]Methionine	830	101·2±7·2 (6)	111·8±8·8 (6)
Algal ^{14}C-labelled protein hydrolysate	2720	99·3±4·6 (6)	93·5±4·1 (6)

the process of transamination is one that occurs outside of the mitochondrion, while the enzymes of the tricarboxylic acid cycle which are necessary for the formation of, for example, α-oxoglutarate from glucose or pyruvate, are situated within the mitochondrion, the difference between the behaviour of alanine and of glutamic acid might be explicable.

It is also possible that in the presence of pyruvate there is appreciable fixation of pyruvate to form oxaloacetate (Ochoa, 1945). The oxaloacetate might then be transaminated to aspartic acid, and this unlabelled aspartic acid would dilute the [^{14}C]-aspartic acid entering the tissue. Incorporation of radioactivity would therefore be less. We have investigated this possibility in more detail (Manchester & Young, 1958).

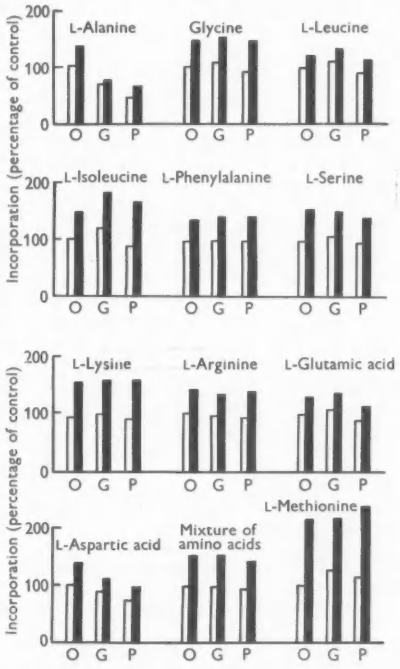

Fig. 1. Effect of insulin, glucose and pyruvate on incorporation *in vitro* of radioactive material from labelled amino acids into protein of normal rat diaphragm. The figures on the ordinate express incorporation as a percentage of that found in the absence of insulin, glucose and pyruvate. Open blocks represent incorporation in the absence of insulin, solid blocks incorporation in the presence of insulin. The letter O signifies that no oxidizable substrate had been added to the medium, G that glucose was present and P that pyruvate was present.

Effect of insulin on protein metabolism. Administration of insulin to a normal animal lowers the plasma level of amino acids (Luck, Morrison & Wilbur, 1928; Kiech & Luck, 1928; Kerr & Krikorian, 1929), and injection of insulin suppresses the increase of amino acid level in plasma that occurs after evisceration (Frame & Russell, 1946; Ingle, Prestrud & Nezamis, 1947). These observations suggest that insulin influences the nitrogen metabolism of extra-hepatic tissue. Frame & Russell (1946) and Ingle *et al.* (1947) found that this action of insulin was not related to the induced changes in blood-glucose level, though Flock, Block, Mann, Grindlay & Bollman (1952) found that the infusion of glucose could suppress the rise of the plasma amino acid level after hepatectomy.

It has been known for many years that administration of insulin stems the loss of nitrogen from the diabetic animal and induces a positive nitrogen balance. This could be due to either a decreased breakdown or an increased formation of protein in the presence of insulin. In so far as experiments with isolated diaphragm are a measure of what takes place *in vivo*, our results clearly indicate that insulin stimulates the transfer of the carbon of amino acid from extracellular fluid into protein.

Krahl (1953) found that incorporation of ^{14}C from [^{14}C]glycine into glutathione and protein in liver and into diaphragm protein, in tissue removed from the fasting rat, is stimulated by addition of insulin or of glucose, and that in the presence of glucose insulin has no stimulating action. He has suggested therefore (Krahl, 1956) that insulin stimulates peptide synthesis by favouring carbohydrate utilization, a process which would make available more energy and possibly more pentose for the synthesis of ribonucleic acid and protein. But we regard as more significant the observations of Sinex *et al.* (1952), Krahl (1953) and ourselves, that incorporation *in vitro* of radioactivity from [^{14}C]amino acid into protein of normal rat diaphragm is stimulated by insulin in the absence of added carbohydrate, i.e. in circumstances where the action of insulin on carbohydrate metabolism is minimal. These observations suggest that insulin stimulates incorporation of radioactivity by a process which is largely or wholly independent of a stimulation of carbohydrate metabolism. The fact that incorporation of radioactivity of amino acids into protein, and its enhancement in the presence of insulin, is unaffected by addition of glucose or pyruvate adds support to this view.

Ketterer *et al.* (1957) suggested the possibility of a competition between carbohydrate metabolism and protein synthesis with respect to the action of insulin. Circumstances in which insulin promotes carbohydrate utilization could, according to this

view, lead to a diminution of its action on protein synthesis. Our results provide no support for this idea.

Mode of action of insulin on protein synthesis. The fact that addition of oxidizable substrates does not accelerate incorporation into protein of radioactivity from [^{14}C]amino acids indicates that in diaphragm from a rat which has been fasted 24 hr. protein formation is unlikely to be limited by availability of energy. Formation of protein might be limited either by the rate at which the protein-synthesizing mechanism can work or by the rate at which amino acids can cross the cell membrane from the extracellular fluid into the muscle cell. The action of insulin could therefore be to stimulate the protein-synthesizing mechanism, or to increase the transfer of amino acids across the cell membrane, or possibly to exert an effect on both processes. Our results do not enable us to differentiate between these two main possibilities, but in view of the evidence obtained by Park, Bornstein & Post (1955), Park & Johnson (1955) and Levine & Golstein (1955) that insulin promotes the utilization of glucose by accelerating its rate of entry into the muscle cell, insulin might be thought to stimulate the entry of amino acids into the cell in a similar fashion.

SUMMARY

1. The effect of addition of insulin, glucose and pyruvate on incorporation *in vitro* into protein of normal rat diaphragm of ^{14}C from [^{14}C]amino acids and of ^{35}S from a [^{35}S]amino acid has been studied.

2. Incorporation of radioactivity from the [^{14}C]amino acids glycine, leucine, isoleucine, phenylalanine, serine, lysine, arginine, glutamic acid and aspartic acid, each studied separately, and from a mixture of [^{14}C]amino acids, was increased by addition of insulin both in the presence and absence of glucose or pyruvate. Neither glucose nor pyruvate greatly affected incorporation in the presence or absence of insulin.

3. Incorporation of ^{35}S from [^{35}S]methionine sulphoxide was accelerated by addition of insulin both in the presence and absence of glucose or pyruvate. Addition of glucose slightly increased incorporation in the absence of insulin, but neither glucose nor pyruvate affected incorporation in the presence of insulin.

4. Incorporation of ^{14}C from alanine was increased in the presence of insulin in the absence of glucose or pyruvate, but was markedly decreased, in the absence of insulin, by addition of pyruvate or, to a less extent, of glucose; in the presence of these substrates insulin did not stimulate incorporation. The possibility is discussed that this behaviour of added alanine is attributable to the formation in muscle of alanine from pyruvate or glucose by a process of transamination.

We are grateful to Dr D. Gross for a gift of L-[^{35}S]-methionine sulphoxide and to Mr W. A. Broom of Boots Pure Drug Co. for a gift of insulin. This work has been greatly helped by a generous grant from the British Diabetic Association for the purchase of radioactive amino acids. Other costs were in part defrayed from an expenses grant from the Medical Research Council to one of us (F.G.Y.). The Medical Research Council also provided a personal grant for K.L.M. To the British Diabetic Association and to the Medical Research Council we wish to express our thanks for assistance in this way, which has made this research possible.

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Synthesis of ε-Aspartyl-lysines and of *iso*Hexylamides of Aspartic Acid

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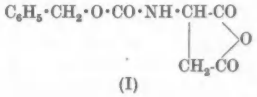
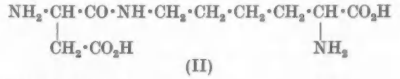
Lockhart & Abraham (1956) found that two compounds which appeared to be aspartyl-lysine and aspartyl(isoleucyl)-lysine respectively were formed when bacitracin A was hydrolysed with 11N-hydrochloric acid at 80°. The aspartic acid residue in these compounds had the L-configuration and was linked to the ε-amino group of L-lysine. However, both compounds carried a net positive charge at pH 7 and this raised the question whether they were simple peptides. The synthesis of peptides in which aspartic acid is joined through its α- and β-carboxyl group respectively to the ε-amino group of lysine has therefore been undertaken. For purposes of comparison the α- and β-*iso*hexylamides of aspartic acid have also been synthesized. L-Aspartic acid and L-lysine have been used in this work but no attempt has been made to discover whether significant racemization occurs in the course of any of the reactions.

It seemed possible that the reaction of *N*-benzyloxycarbonyl-L-aspartic anhydride (I) with the copper complex of L-lysine or with α-toluene-*p*-sulphonyl-L-lysine ester (α-tosyl-L-lysine ester) would provide a simple route to both ε-*N*-(α-L-aspartyl)-L-lysine (II) and ε-*N*-(β-L-aspartyl)-L-lysine (III). The anhydride (I) was used by Bergmann & Zervas (1932) in peptide synthesis and was later shown to yield a mixture of α- and β-aspartyl derivatives on reaction with several amino acid esters (LeQuesne & Young, 1952; John & Young, 1954).



(I)



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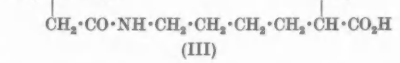

(III)

Interaction of the anhydride with the copper complex of L-lysine in aqueous dioxan, followed by removal of copper and of the benzyloxycarbonyl group, led to a product from which only one compound, subsequently shown to be ε-(α-L-aspartyl)-L-lysine, was obtained by crystallization from water. The non-crystalline material from the mother liquors contained some of the β-aspartyl isomer.

Reaction of the anhydride with α-tosyl-L-lysine ethyl ester in a mixture of ethyl acetate and aqueous potassium bicarbonate appeared to occur in the expected fashion, but difficulty was experienced in the removal of the ethyl ester group. This difficulty was avoided by use of the corresponding benzyl ester and subsequent removal of the benzyl and benzyloxycarbonyl group together by catalytic hydrogenation. The final product was shown by electrophoresis on paper to have no significant net charge at pH 7·0 and 5·0 and to yield equivalent amounts of aspartic acid and lysine on hydrolysis. When subjected to electrophoresis on paper at pH 2·3, however, it was resolved into two components which migrated towards the cathode at different rates. The faster-moving component was assumed to be the α-aspartyl peptide (II) and the slower moving the β-aspartyl peptide (III), since the free β-carboxyl group of the former should be a weaker acid than the corresponding α-carboxyl group of the latter. The faster-moving component migrated at the same rate as the peptide obtained from the copper complex of lysine.

This assumption was shown to be correct when the two isomers were synthesized by unambiguous routes. The syntheses involved the coupling, by the mixed-anhydride method (Vaughan, 1951), of an α-tosyl-L-lysine benzyl ester with the β- and α-benzyl esters respectively of *N*-benzyloxycarbonyl-L-aspartic acid. The benzyl and benzyloxycarbonyl groups were removed from the products by catalytic hydrogenation and the tosyl group by sodium in liquid ammonia. ε-(α-L-Aspartyl)-L-lysine, obtained from the β-benzyl aspartate, migrated as a single compound, and at the same rate as the faster-moving of the two isomers prepared earlier, when subjected to electrophoresis on paper at pH 2·3. ε-(β-L-Aspartyl)-L-lysine, obtained from the α-benzyl aspartate, proved to have the same mobility at pH 2·3 as the slower-moving isomer. It was contaminated, however, with a trace of the α-aspartyl peptide. This indicated that the method used for the preparation of α-benzyl

N-benzyloxycarbonylaspartate (Bergmann, Zervas & Salzmann, 1933) had given a product containing a small proportion of the β-benzyl ester.

The results of electrometric titrations of peptides (II) and (III) are shown in Fig. 1. ε-(α-L-Aspartyl)-L-lysine contained ionizable groups with pK_a values of $<2{\cdot}7$, 3·4, 8·0 and 9·9. These values could be assigned to the α-carboxyl group of the lysine residue, the β-carboxyl group of the aspartic acid residue, the amino group of the aspartic acid residue, and the α-amino group of the lysine residue respectively. In ε-(β-L-aspartyl)-L-lysine the corresponding pK_a values were $<2{\cdot}5$, 2·8, 9·1 and 10·1. As expected, the second carboxyl of the β-aspartyl isomer was a stronger acid than that of the α-isomer, whereas the first amino group of the α-isomer (being in the α position to a peptide bond) was a weaker base than the first amino group of the β-isomer.

The titration curves of the two ε-aspartyl-lysines accounted for the results obtained when the compounds were subjected to electrophoresis on paper in different buffers. At pH 7·0 and pH 5·0 both peptides behaved as neutral substances. At pH 2·3 ε-(α-L-aspartyl)-L-lysine migrated 11 cm. towards the cathode under the conditions used (14 v/cm. for 2·5 hr.) and ε-(β-L-aspartyl)-L-lysine migrated 8 cm. towards the cathode. At pH 9·1 the α-isomer migrated 5·6 cm. towards the anode and the β-isomer 2·5 cm. towards the anode.

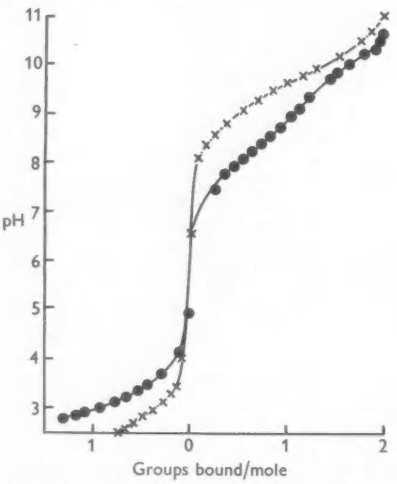

Fig. 1. Electrometric titrations in water at 20° with 0·1 N-HCl and 0·1 N-NaOH. ●, ε-*N*-(α-L-Aspartyl)-L-lysine (1·5 mg./ml.); ×, ε-*N*-(β-L-Aspartyl)-L-lysine (1·8 mg./ml.).

The two ε-aspartyl-lysines were not separated by paper chromatography in butanol–acetic acid–water, both compounds showing an R_F close to that of lysine. In a phenol–water–ammonia system both compounds showed an R_F of 0·56. Attempts to separate the compounds by chromatography in other solvent systems (see Experimental) were also unsuccessful.

ε-(α-Aspartyl)-lysine gave a biuret colour, whereas the β-isomer did not. Liwschitz & Zilkha (1954) have previously reported that the biuret reaction can be used to distinguish between α- and β-aspartyl peptides. When ε-(α-aspartyl)-lysine was allowed to react with ninhydrin under the conditions used by Van Slyke, Dillon, MacFadyen & Hamilton (1941), 1·49 molecular proportions of carbon dioxide were evolved after 6 min., but this value rose to 1·83 when heating was continued for 25 min. This is consistent with the finding of John & Young (1954) that α-aspartylvaline evolved a significant amount of carbon dioxide after 7 min. and about one molecular proportion after 20 min.

The α- and β-*iso*hexylamides of aspartic acid were synthesized by three methods, two of which were similar to methods used for the preparation of ε-aspartyl-lysines. The reaction of benzyloxycarbonyl-L-aspartic anhydride with *iso*hexylamine led to a mixture of α- and β-*iso*hexylamides, the α-isomer predominating. The β-*iso*hexylamide was obtained from α-benzyl *N*-benzyloxycarbonyl-L-aspartate and *iso*hexylamine by the mixed-anhydride method; the presence of some α-isomer in the mother liquors from the crystalline product again indicated that the α-benzyl *N*-benzyloxycarbonyl aspartate used was contaminated with a small amount of the corresponding β-benzyl ester. The third procedure was based on the method of Liwschitz & Zilkha (1955) for synthesizing β-aspartyl peptides. *N-iso*Hexylmaleamic acid (IV) was prepared

$$\begin{matrix} CH\cdot CO\cdot NH\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH(CH_3)_2 \\ \| \\ CH\cdot CO_2H \end{matrix}$$

(IV)

from maleic anhydride and *iso*hexylamine under conditions similar to those used by King, Clark-Lewis, Wade & Swindin (1957) for obtaining maleamic acids. Addition of benzylamine to the double bond of (IV) gave a mixture of the α- and β-*iso*hexylamides of *N*-benzyl-DL-aspartic acid, but the two isomers had widely different solubilities in hot dioxan and were readily separated. Removal of the benzyl group from the two compounds by catalytic hydrogenation gave the corresponding *iso*hexylamides of aspartic acid.

The α- and β-*iso*hexylamides of aspartic acid were readily separated by electrophoresis on paper

(14 v/cm. for 2·5 hr.) at pH 2·3, the former migrating 8·7 and the latter 4·2 cm. towards the cathode. The two compounds could also be distinguished on paper chromatograms run with butanol–acetic acid–water, the R_F of the α-isomer being 0·80 and that of the β-isomer 0·75.

EXPERIMENTAL

L-Aspartic acid and L-lysine were obtained from British Drug Houses Ltd., and *iso*hexylamine from L. Light and Co. Ltd., Colnbrook, Bucks. Analyses were by Weiler and Strauss, Oxford.

Electrometric titrations. These were carried out in the manner described by Newton & Abraham (1953).

Paper chromatography and electrophoresis of peptides. Paper chromatograms were run on Whatman no. 1 paper. The solvent systems used were butanol–acetic acid–water (Woiwod, 1949), phenol–water–NH_3 (Underwood & Rockland, 1954), triethylamine–acetone–water (Wright & Stadtman, 1956), *tert.*-butanol–methanol–water (Boissonnas, 1950) and butan-2-one–propionic acid–water (Clayton & Strong, 1954).

Electrophoresis on Whatman no. 1 paper was carried out as described by Newton & Abraham (1954). The buffers used were 0·05 M-collidine acetate (0·05 M to acetate, pH 7), pyridine acetate (0·05 M to acetate, pH 5), 10% (v/v) acetic acid (pH 2·3), and 0·02 M-sodium borate, pH 9·1. The potential gradient was 14 v/cm. and electrophoresis was continued for 2·5 hr. Spots were located by spraying with ninhydrin.

2:4-Dinitrophenyl (DNP) derivatives. These were prepared by the method of Sanger & Thompson (1953) under the conditions used by Lockhart & Abraham (1956). DNP-aspartic acid was identified by chromatography on paper in the system of Blackburn & Lowther (1951). α-DNP-lysine and ε-DNP-lysine were separated by electrophoresis on paper at pH 9·1 as described by Lockhart & Abraham (1956).

N-Benzyloxycarbonyl-L-aspartic acid. This was prepared by a modification of the method of Bergmann & Zervas (1932), for which we are indebted to Dr W. D. John and Dr G. T. Young. L-Aspartic acid (2·0 g.) was dissolved in a solution of NaOH (1·85 g.) in water (6·1 ml.). The solution was covered with a layer of ether, stirred vigorously and cooled to 0° in ice. Benzyl chloroformate (2·5 ml., containing 1·14 g. of chloroformate/ml.) was added in small portions over 30 min. The mixture was stirred for a further 30 min. at 0° and for 2 hr. at room temperature. The ethereal layer was removed and the aqueous layer washed twice with 10 ml. portions of ether. The solution was filtered and made acid to Congo red with 11 N-HCl, when an oil separated. The oil and supernatant solution were shaken and traces of ether removed *in vacuo* (water pump). The oil then solidified and the solid product was collected and dried. Yield 66%, m.p. 117°.

N-Benzyloxycarbonyl-L-aspartic anhydride. This was prepared by the method of John & Young (1954), but the product was not washed with cold ether before crystallization. Needles, m.p. 113°, were obtained (John & Young give m.p. 111°) in 85% yield (Found: C, 57·6; H, 4·3; N, 5·4. Calc. for $C_{12}H_{11}O_5N$: C, 57·8; H, 4·4; N, 5·6%).

Dibenzyl N-benzyloxycarbonyl-L-aspartate. The method of Berger & Katchalski (1951) was used. Distillation under high vacuum (0·05 mm.) was required to remove excess of benzyl alcohol from the reaction mixture. Otherwise the product would not crystallize on trituration with light petroleum (b.p. 40–60°).

β-Benzyl N-benzyloxycarbonyl-L-aspartate. The method of Berger & Katchalski (1951) was followed, but very small yields of the product were obtained and 75% of the starting material was recovered. Somewhat higher, but still small, yields were obtained when the reaction was carried out at 37° instead of at room temperature. The product crystallized from a small volume of benzene, giving needles, m.p. 108–109°.

α-Benzyl N-benzyloxycarbonyl-L-aspartate. The method of Bergmann *et al.* (1933) was used.

ε-N-Benzyloxycarbonyl-L-lysine. This was prepared by the method of Neuberger & Sanger (1943). The lysine copper complex was isolated by precipitation with acetone as a pale-blue amorphous powder. It was kept as such for use in future experiments.

ε-N-Benzyloxycarbonyl-α-tosyl-L-lysine. ε-Benzyloxycarbonyl-L-lysine (2·0 g.) was dissolved in 2 N-NaOH (46 ml.) and shaken vigorously for 4 hr. with finely powdered toluene-*p*-sulphonyl (tosyl) chloride (8 g.). The solution was filtered, the residual solid well washed with 2 N-NaOH, and the combined filtrate and washings were acidified with 2 N-H_2SO_4. The liberated oil was extracted from the mixture with ether (3 × 1 vol.), the ethereal extracts were dried over anhydrous Na_2SO_4, and the ether was removed *in vacuo*. The residue (2·5 g.) was crystallized from benzene, giving fine needles. *ε-N-Benzyloxycarbonyl-α-tosyl-L-lysine*, which appeared to contain benzene of crystallization, had m.p. 126° (Found: C, 62·8; H, 6·2; N, 5·1; S, 5·7. $C_{21}H_{26}O_6N_2S$ requires C, 58·1; H, 6·0; N, 6·4; S, 7·4. $C_{21}H_{26}O_6N_2S,C_6H_6$ requires C, 63·3; H, 6·3; N, 5·4; S, 6·3%).

α-N-Tosyl-L-lysine. *ε-N*-Benzyloxycarbonyl-α-tosyl-L-lysine (14·2 g.) was dissolved in 80% (v/v) methanol (200 ml.) to which acetic acid (1·0 ml.) was added. Pd–charcoal (1·0 g., 10% of Pd, L. Light and Co. Ltd.) was added and the mixture hydrogenated until no more CO_2 was evolved. The product, which had precipitated from solution, was extracted from the charcoal with boiling water and the solution filtered hot. The mother liquors and washings were evaporated to dryness in the rotary evaporator (Craig, Gregory & Hausmann, 1950) at 30° and the product (7·5 g., 77%) obtained as a mass of minute hair-like crystals. It was insoluble in hot ethanol or benzene, but was appreciably soluble in cold water. *α-Tosyl-L-lysine* did not melt at temperatures up to 220°, behaved as a neutral substance on paper electrophoresis at pH 7·0, and ran with R_F 0·81 on chromatography in butanol–acetic acid–water.

α-Tosyl-L-lysine ethyl ester hydrochloride. α-Tosyl-L-lysine (480 mg.) was refluxed for 3 hr. with anhydrous ethanol (6·5 ml.) saturated with dry HCl. The solvent and HCl were removed *in vacuo*, leaving a colourless oil which slowly crystallized at room temperature. The product (445 mg., yield 76%) was recrystallized from ethanol–ether as fine needles. *α-Tosyl-L-lysine ethyl ester hydrochloride* had m.p. 180·5° (Found: C, 48·9; H, 7·0; N, 7·4; S, 8·5; Cl, 9·3. $C_{15}H_{25}O_4N_2SCl$ requires C, 49·4; H, 6·9; N, 7·7; S, 8·9; Cl, 9·7%).

α-Tosyl-L-lysine benzyl ester hydrochloride. Freshly distilled benzyl alcohol (b.p. 86°/7 mm. Hg) was saturated

with anhydrous HCl, and the solution (6·0 ml.) added to α-tosyl-L-lysine (0·4 g.). The mixture was heated on a boiling-water bath under a reflux condenser closed by a tube of anhydrous $CaCl_2$. After 3 hr. the mixture was distilled under vacuum to remove excess of benzyl alcohol. The residual oil slowly crystallized after standing for 2 hr. at room temperature. The solid was recrystallized from ethanol–ether, giving small colourless needles, m.p. 164–165° (0·45 g., yield 79%). This material was dissolved in a small volume of water and conc. HCl added. The ester hydrochloride was precipitated as fine colourless needles and was collected, washed with 2N-HCl, then with acetone, and finally dried in a vacuum desiccator in the presence of NaOH. *α-Tosyl-L-lysine benzyl ester hydrochloride* had m.p. 170° (Found: C, 55·9; H, 6·5; N, 6·3; S, 7·5; Cl, 8·6. $C_{20}H_{27}O_4N_2SCl$ requires C, 56·3; H, 6·3; N, 6·5; S, 7·5; Cl, 8·3%).

An attempt to prepare this substance by the method of Erlanger & Hall (1954), with polyphosphoric acid as condensing agent, gave a very poor yield.

ε-L-Aspartyl-L-lysine. α-Tosyl-L-lysine benzyl ester hydrochloride (0·1 g.) was dissolved in water (2·5 ml.) together with $KHCO_3$ (0·2 g.), and the solution covered with a layer of ethyl acetate (4·0 ml.). A solution of benzyloxycarbonyl-L-aspartic anhydride (0·12 g.) in ethyl acetate (2 ml.) was added and the mixture stirred vigorously for 5·5 hr. The aqueous layer was separated, washed with ethyl acetate and acidified with 2N-HCl. The liberated oil was extracted with three portions of ethyl acetate (each 1 ml.), the solvent evaporated and the residue dried in a vacuum desiccator. This product (0·14 g.) was dissolved in 65% (v/v) ethanol (9·0 ml.) to which acetic acid (0·2 ml.) was added. The solution was hydrogenated in the presence of Pd–charcoal (50 mg.) until no more CO_2 was evolved. The charcoal was filtered off and washed with water and the filtrate and washings were evaporated to dryness in a rotary evaporator. A small sample of the residue was subjected to paper electrophoresis at pH 7 and appeared to contain mainly aspartyl-α-tosyl-lysine, together with some aspartic acid and traces of lysine and α-tosyl-lysine. The main part of the product was dissolved in liquid NH_3 (about 5 ml.) and Na added until a permanent blue colour resulted. The colour was discharged with NH_4Cl and the NH_3 allowed to evaporate. The residue was dissolved in water, the pH adjusted to 6·0 with N-HCl, and the solution extracted with ether. This removed toluene-*p*-sulphonamide. The remaining aqueous phase was submitted to ionophoresis in a four-compartment cell (Synge, 1951) until the current fell to 25 mA at 500 V. The solution from the specimen compartment was then evaporated to dryness in a rotary evaporator. Paper electrophoresis showed that the amorphous residue (13·7 mg.) was mainly neutral at pH 7, but contained a trace of lysine.

The product was shown to consist of ε-aspartyl-lysine by hydrolysis with 6N-HCl at 105° for 24 hr., after which only aspartic acid and lysine (in approximately equal amounts) were revealed by electrophoresis on paper, and by the preparation of its DNP derivative, which gave DNP-aspartic acid and α-DNP-lysine on hydrolysis. However, electrophoresis on paper in 10% (v/v) acetic acid showed that the product was a mixture of ε-(α-aspartyl)-lysine and ε-(β-aspartyl)-lysine.

ε-(α-L-Aspartyl)-L-lysine. (*a*) Prepared from lysine copper complex (0·67 g., 1·0 mol. prop.) and benzyloxycarbonylaspartic anhydride (1·12 g., 1·5 mol. prop.). The copper complex was dissolved in a mixture of water (7 ml.) and dioxan (8 ml.) and the solution cooled to 0° in ice. The anhydride was dissolved in dioxan (4 ml.) and added in portions, with stirring, over 0·5 hr., together with N-NaOH (3·0 ml.). The homogeneous mixture was kept at 0° for a further 1·5 hr. The solution was adjusted to pH 7 with N-NaOH and allowed to stand overnight. After acidification with acetic acid, the solution was extracted 6 times with 30 ml. of ether to remove dioxan, and the aqueous phase was then saturated with H_2S. The precipitate of CuS was centrifuged off and the solution boiled and aspirated until no more H_2S could be detected. The clear solution was hydrogenated in the presence of Pd–charcoal until no more CO_2 was evolved, filtered and submitted to ionophoresis in the 4-compartment cell. When the current had fallen to a minimum (25 mA at 500 V) the contents of the specimen compartment were evaporated to dryness, and the residue (0·39 g.) crystallized from water (1·0 ml.). Fine white needles (98 mg.) were obtained, which appeared to be pure ε-(α-aspartyl)-lysine. No β-isomer was detected by electrophoresis in 10% (v/v) acetic acid. *ε-(α-L-Aspartyl)-L-lysine* did not melt below 300° and, after drying *in vacuo* at 100°, rapidly took up water from the atmosphere to the extent of $2H_2O$/molecule: for the dihydrate $[\alpha]_D^{20} + 13·2°$ in 0·5N-HCl (*c*, 1·36) (Found: C, 40·5; H, 7·6; N, 14·2. $C_{10}H_{19}O_5N_3,2H_2O$ requires C, 40·4; H, 7·7; N, 14·1%).

(*b*) Prepared by the mixed-anhydride method. β-Benzyl *N*-benzyloxycarbonyl-L-aspartate (0·21 g.) was dissolved in dry $CHCl_3$ (10 ml.) and one equivalent (0·084 ml.) of triethylamine added. After being shaken for a few minutes the solution was cooled to 0° and one equivalent of freshly distilled ethyl chloroformate (0·057 ml.) was added. The solution was kept at 0° for 45 min. to allow the formation of the mixed anhydride to go to completion.

α-Tosyl-L-lysine benzyl ester hydrochloride (0·25 g.) was converted into the free ester by the method of Hillmann (1946). The $CHCl_3$ solution (0·75 ml.) of the ester was added to that of the mixed anhydride, and the mixture shaken well and left to stand overnight at room temperature. The solution was washed once with water, twice with $NaHCO_3$ solution, and finally twice with water. The $CHCl_3$ layer was dried with anhydrous $MgSO_4$, filtered and diluted with a large volume of light petroleum (b.p. 60–80°). The precipitate of fully protected peptide (0·25 g.) had m.p. 120–122°.

The benzyl and benzyloxycarbonyl groups were removed from this material by catalytic hydrogenation (Pd-charcoal) in 75% (v/v) methanol containing 1·5% (v/v) acetic acid and the tosyl group was then removed with Na in liquid NH_3 in the usual manner. The product was subjected to ionophoresis in the 4-compartment cell until the current fell to 25 mA at 500 V. Evaporation of the solution from the specimen compartment yielded 54 mg. of ε-(α-aspartyl)-lysine. This showed only a single spot when subjected to paper electrophoresis in 10% (v/v) acetic acid, or in borate buffer (pH 9·1). It was indistinguishable, under these conditions, from the product obtained by method (*a*).

ε-(β-L-Aspartyl)-L-lysine. This was prepared in a similar way to the α-isomer by the mixed-anhydride method, α-benzyl *N*-benzyloxycarbonyl-L-aspartate being used. The product was shown by paper electrophoresis in 10% (v/v) acetic acid to be mainly the β-isomer but to be contaminated with a trace of α-isomer.

*β-iso**Hexylamide of* L-*aspartic acid*. This was prepared by the mixed-anhydride method as above from α-benzyl *N*-benzyloxycarbonyl-L-aspartate and *iso*hexylamine. Catalytic hydrogenation (Pd–charcoal) of the protected amide in 80% (v/v) ethanol gave the free amide (71 mg., yield 47%). This product was crystallized from water and gave fine rosettes of needles. On paper electrophoresis in 10% (v/v) acetic acid the crystals behaved as though they were pure β-amide (single brown spot with ninhydrin). A second crop of crystals, obtained on partial evaporation of the mother liquors, was also pure β-isomer, but third and fourth crops contained about 20 and 40% respectively of the α-amide (purple spot with ninhydrin).

The *β-iso**hexylamide of* L-*aspartic acid* had m.p. 208° (decomp.) (Found: C, 55·3; H, 9·1; N, 13·2. $C_{10}H_{20}O_3N_2$ requires C, 55·5; H, 9·3; N, 13·0%).

N-iso*Hexylmaleamide*. A solution of maleic anhydride (1·0 g.) and *iso*hexylamine (1·0 g., 1·32 ml.) in acetic acid (20 ml.) was allowed to stand at room temperature overnight. The acetic acid was removed by distillation *in vacuo* and the brownish yellow oil partly solidified on cooling. It was dissolved in methanol and precipitated as an oil by the addition of a large volume of water. The oil solidified on cooling in ice and the solid was collected and dried (yield 1·46 g., 72·5%), m.p. 83–84°. The solid was crystallized with difficulty from ether–light petroleum (b.p. 60–80°). Four recrystallizations, in which only the first crop of colourless needles from each process were taken, gave a final product of constant m.p. *N*-iso*Hexylmaleamide* had m.p. 110° (Found: C, 60·6; H, 8·6; N, 7·0. $C_{10}H_{17}O_2N$ requires C, 60·3; H, 8·6; N, 7·0%).

The method of Liwschitz & Zilkha (1955) for the preparation of maleamic acids, in which ether was used as a solvent, gave a product which was slightly easier to purify, but only in 32% yield.

*α- and β-iso**Hexylamides of* N-*benzyl*-DL-*aspartic acid*. *N-iso*Hexylmaleamide (1·05 g.) was dissolved in redistilled dry dioxan (10 ml.) and freshly distilled benzylamine (0·8 g.) was added. The solution was refluxed on an oil bath at 120°. After about 25 min. a white suspension formed. The mixture was refluxed for a further 30 min., cooled, and the solid collected. It was washed with dioxan and dried, yielding 0·57 g., m.p. 198° (decomp.). The slightly turbid filtrate was refluxed for a further hour, but no more precipitate formed. The dioxan was distilled off, leaving an oily residue. Acetone (10 ml.) was added and, on shaking, the oil solidified. The white amorphous powder was collected, washed with acetone and dried, yielding 0·63 g., m.p. 150–152°.

Both products were crystallized from 30% (v/v) methanol. The *β-iso**hexylamide of* N-*benzyl*-DL-*aspartic acid*, which was insoluble in boiling dioxan, gave rosettes of needles, m.p. 199° (decomp.) (Found: C, 66·8; H, 8·3; N, 8·8. $C_{17}H_{26}O_3N_2$ requires C, 66·7; H, 8·5; N, 9·1%). The *α-iso**hexylamide of* N-*benzyl*-DL-*aspartic acid*, which was readily soluble in boiling dioxan, crystallized in small leaflets, m.p. 157·5° (Found: C, 67·0; H, 8·4; N, 8·8. $C_{17}H_{26}O_3N_2$ requires C, 66·7; H, 8·5; N, 9·1%).

The oily residues from the preparation of N-*iso*hexylmaleamide gave a good yield of the *β-iso*hexylamide of *N*-benzyl-DL-aspartic acid when treated with benzylamine.

*α- and β-iso**Hexylamides of* DL-*aspartic acid*. The benzyl group was removed from the α- and *β-iso*hexylamides of *N*-benzyl-DL-aspartic acid by catalytic hydrogenation at 75° in 66% (v/v) ethanol in the presence of Pd–charcoal (10% Pd) (cf. Velluz, Amiard & Heymès, 1955). The solution was filtered hot, the Pd–charcoal washed once with hot 66% (v/v) ethanol and the filtrate and washings evaporated to dryness in a rotary evaporator. The residue was crystallized from slightly aqueous ethanol. The identity of the products was confirmed by examination on paper electrophoresis in 10% (v/v) acetic acid and by paper chromatography in butanol–acetic acid–water. The *β-iso*hexylamide of *N*-benzylaspartic acid gave the *β-iso*hexylamide of DL-aspartic acid, m.p. 208° (decomp.) (brown spot with ninhydrin). The *α-iso*hexylamide of *N*-benzylaspartic acid gave the *α-iso**hexylamide of* DL-*aspartic acid*, m.p. 197° (purple spot with ninhydrin) (Found: C, 55·4; H, 9·0; N, 11·7; 14·8. $C_{10}H_{20}O_3N_2$ requires C, 55·5; H, 9·3; N, 13·0%).

SUMMARY

1. ε-*N*-(α-L-Aspartyl)-L-lysine has been obtained by interaction of *N*-benzyloxycarbonyl-L-aspartic anhydride with the copper complex of L-lysine.

2. ε-*N*-(α-Aspartyl)-L-lysine and the corresponding β-aspartyl-lysine have been synthesized by the mixed-anhydride method from α-toluene-*p*-sulphonyl-L-lysine benzyl ester and the β- and α-benzyl esters respectively of *N*-benzyloxycarbonyl-L-aspartic acid.

3. The *β-iso*hexylamide of L-aspartic acid has been synthesized by the mixed-anhydride method from α-benzyl *N*-benzyloxycarbonyl-L-aspartate and *iso*hexylamine. The α- and *β-iso*hexylamides of DL-aspartic acid have been synthesized from *N-iso*hexylmaleamic acid.

4. The two ε-aspartyl-lysines are readily separated by electrophoresis on paper at pH 2·3 or 9·1, but not by paper chromatography in a number of solvent systems. The two *iso*hexylamides of aspartic acid can be separated in paper chromatograms run in butanol–acetic acid–water.

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Formation of ε-(Aminosuccinyl)-lysine from ε-Aspartyl-lysine from Bacitracin A, and from the Cell Walls of Lactobacilli

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Work in two Laboratories has led to the conclusion that the polypeptide antibiotic bacitracin A contains the sequence L-Asp $\overset{\varepsilon}{\rightarrow}$ L-Lys $\overset{\alpha}{\leftarrow}$ L-Ileu, in which $\rightarrow$ denotes a C–N bond (Lockhart & Abraham, 1954*a*, *b*, 1956; Craig, Hausmann & Weisiger, 1954; Theodoropoulos & Craig, 1956). Part of the evidence for this sequence is based on the analysis of two products of hydrolysis of bacitracin A which are strikingly resistant to acid. One of these products appeared to consist only of lysine and aspartic acid residues and the other of lysine, aspartic acid and isoleucine residues (Lockhart & Abraham, 1956). Nevertheless, both products were more basic than was to be expected if they were peptides containing two amino groups and two carboxyl groups. Further information about their structures was therefore desirable.

Experiments recorded in this paper provide an explanation of the anomalous behaviour of the products containing lysine and aspartic acid which are formed from bacitracin A. Synthetic ε-(α-L-aspartyl)-L-lysine and ε-(β-L-aspartyl)-L-lysine (Swallow, Lockhart & Abraham, 1958) are both different from the 'Asp.Lys' found in hydrolysates of bacitracin A. However, both synthetic peptides yield a derivative of aminosuccinimide, ε-(aminosuccinyl)-lysine (I), which appears to be identical with the product from the antibiotic, on treatment with concentrated hydrochloric acid at 80°. A similar type of cyclization has been found to occur with the α- and β-*iso*hexylamides of aspartic acid (Swallow *et al.* 1958) and (less readily) with α-(α-L-aspartyl)-L-lysine and α-(β-L-aspartyl)-L-lysine which have been synthesized by Pimlott & Young (unpublished work).

After this work had begun Cummins & Harris (1956) reported that hydrolysis of the cell walls of certain lactobacilli yielded a substance which was composed largely of lysine and aspartic acid but which resisted treatment with 6N-hydrochloric acid at 100°. The properties of this substance raised the question whether it was related to the cyclic compound from bacitracin A and from the ε-aspartyl-lysines (Swallow & Abraham, 1957). A direct comparison of the two products has now indicated that they are identical or differ only in optical configuration.

Brief accounts of some of the work described here have already been given (Swallow & Abraham, 1957; Abraham, 1957).

METHODS

Materials

ε-Aspartyl-lysines. The α- and β-isomers were prepared as described by Swallow *et al.* (1958).

α-Aspartyl-lysines. The two isomers were kindly provided by Miss P. Pimlott and Dr G. T. Young.

N-Benzyloxycarbonyl-L-aspartic anhydride. This was prepared by the method of John & Young (1954).

α- and β-isoHexylamides of aspartic acid. These were prepared by the method of Swallow *et al.* (1958).

Bacitracin. The bacitracin used (lot B 55–13, 68 i.u./mg.) was supplied by the Commercial Solvents Corporation, Terre Haute, Indiana, U.S.A.

Partial hydrolysate of bacitracin. A solution of bacitracin in 11N-HCl (40 ml./g.) was kept at 80° for 43 hr. in a sealed flask. The hydrolysate was evaporated to dryness in a rotary evaporator (Craig, Gregory & Hausmann, 1950). The residue was dissolved in water and evaporation repeated twice to remove HCl. The pattern obtained when

this hydrolysate was subjected to electrophoresis on paper in 0·05M-collidine acetate (pH 7) was similar to that obtained earlier with a partial hydrolysate of bacitracin A (Lockhart & Abraham, 1956).

Partial hydrolysate of ε-aspartyl-lysine. A solution of ε-aspartyl-lysine in 11N-HCl (20 ml./g.) was kept at 80° for 24 hr. The hydrolysate was treated in a similar manner to the partial hydrolysate of bacitracin.

Licheniformin. We are grateful to Dr R. K. Callow and Dr T. S. Work for samples of licheniformins A, B and C.

Bacterial cell walls. We have to thank Dr C. S. Cummins and Dr H. Harris for a sample of the cell walls of *Lactobacillus brevis* and Mrs K. Crawford for the preparation of cell walls of *Bacillus licheniformis* by the method of Cummins & Harris (1956).

Hydrolysis and identification of peptides

Peptides were hydrolysed in sealed Pyrex tubes, usually in 0·5 ml. of acid under conditions described in the text. Acid hydrolysates were evaporated to dryness *in vacuo* at room temperature over P_2O_5 in the presence of solid NaOH. In some cases hydrolysis was carried out in 0·3N-$Ba(OH)_2$. Most of the Ba^{2+} ions were precipitated from these solutions, as $BaCO_3$, before evaporation *in vacuo*.

Chromatography and electrophoresis on Whatman no. 1 paper were carried out in the systems described by Swallow *et al.* (1958). In general, 20–40 μg. of peptide was applied to the paper.

2:4-Dinitrophenyl (DNP) derivatives were prepared as described by Lockhart & Abraham (1956).

Electrometric titrations were carried out in the manner described by Newton & Abraham (1953).

Semiquantitative determinations of amino acids and peptides separated by paper electrophoresis. The components of some hydrolysates were estimated semiquantitatively, after separation by electrophoresis, by a method similar to that of Peart (1956). Samples of hydrolysate (each 1·7 μl. containing about 30 μg. of product) were spotted at intervals on a single sheet of paper. Known amounts of leucine (2–16 μg.) were also spotted on the same sheet. After electrophoresis in 10% (v/v) acetic acid (14 v/cm., 2·5 hr.) the paper was dried in air at 60°, sprayed on both sides with a solution of ninhydrin in butanol (0·16%, w/v) and heated for 15 min. at 90°. Squares of paper containing the spots were cut out, together with two blank squares. Each square was cut into strips and placed in a small test-tube containing 5 ml. of 50% (v/v) ethanol. The tubes were shaken occasionally during 1 hr. to elute the pigment, and the optical density of each solution was then measured at 570 mμ in a Beckman spectrophotometer. Provided that the amount of amino acid or peptide was not less than 2 μg., the accuracy for duplicate runs was ±10%. The molar colour yields of ε-(α-Asp).Lys, ε-(β-Asp).Lys, ε(aminosuccinyl)-lysine [ε-(Asu).Lys], aspartic acid and lysine were measured against a leucine standard in a similar way and found to be 1·4, 0·9, 0·7, 0·6 and 0·9 respectively. The corresponding values obtained by the usual ninhydrin method in solution (Moore & Stein, 1948) were 1·43, 1·64, 1·08, 0·88 and 1·12 respectively.

Chromatography on ion-exchange resins

Aspartic acid, glutamic acid and phenylalanine were removed from partial hydrolysates of bacitracin by adsorption on a column of Dowex 1 × 10 (200–400 mesh) in the acetate form (Fig. 1*a*) under conditions similar to those used by Hirs, Moore & Stein (1954). Free aspartic acid was removed similarly from the products obtained by treating ε-(α-L-aspartyl)-L-lysine with 11N-HCl at 80°. The ninhydrin colour densities given by samples (0·1 ml.) of fractions from the column were determined by the method of Moore & Stein (1954). Colour densities were measured in an Evans Electroselenium Ltd. (EEL) portable colorimeter.

The fractions containing the basic and neutral amino acids and peptides (except phenylalanine, see Fig. 1*a*) were bulked and evaporated in a rotary evaporator with a bath temperature at 25°. The residue was chromatographed at 22°, as shown in Fig. 1*b*, on a column of Dowex 50 × 4 (200–400 mesh) prepared according to Hirs *et al.* (1954). Elution was begun with 1·0N-HCl. When 185 fractions had been collected, a mixing chamber (250 ml., stirred magnetically) was introduced into the flow system and by means of this the strength of HCl was gradually raised from N to 4N (a further 330 fractions). Ninhydrin colour densities (Moore & Stein, 1954) were determined on samples (0·1 ml.) from every fraction containing ninhydrin-positive material. Further samples (0·5 ml.) were taken from every twentieth fraction for titration of the acid; when the normality of the latter was greater than 2 the samples used for ninhydrin determinations were neutralized with 2N-NaOH.

The fractions within each peak were collected and evaporated to dryness in a rotary evaporator. The identity of the residue was ascertained by electrophoresis on paper in collidine acetate (0·05M to acetate, pH 7·0) and by paper chromatography in butanol–acetic acid–water (Woiwod, 1949).

Continuous paper electrophoresis

In some experiments electrophoresis was carried out on a preparative scale in a Spinco continuous paper electrophoresis apparatus, model C.P. (Beckman Instruments Inc., Spinco Division, California). The buffer used was pyridine acetate (0·05M to acetate, pH 5·0) and the flow down the paper was adjusted so that a spot of acid fuchsin took about 3 hr. to run the length of the paper (33 cm.). A partial hydrolysate of ε-aspartyl-lysine (3·7 mg./ml.), mixed with bromocresol green and methyl red as visible indicators, was applied to the paper at a constant rate of 1 drop/50 sec. A potential of 13 v/cm. was applied horizontally across the paper (width 35 cm.), and the effluent collected from the 32 drip points at the lower end of the sheet. The drip points were numbered from anode (1) to cathode (32). In a continuous run of 6 days the visible indicators, which ran towards the anode, did not change in position.

The amino acid pattern was ascertained by analysis of samples (0·5 ml.) from each drip point. The samples were evaporated to dryness *in vacuo*, the residues each dissolved in 30 μl. of water and spots (3·5 μl.) of the resulting solutions were used for paper electrophoresis at pH 7·0 in 0·05M-collidine acetate.

Synthesis of L-*aminosuccinylisohexylamine (II)*

Benzyloxycarbonyl-L-aspartic anhydride (2·0 g., 1 mol. prop.) was heated in a sealed tube at 130° for 3 hr. with *iso*hexylamine (1·07 ml., 1 mol. prop.). The tube was cooled, opened and reheated for 1 hr. to drive off the water

eliminated in the reaction. The clear yellow–brown syrup was dissolved in ethanol (30 ml.), acidified with acetic acid (1·0 ml.) and hydrogenated in the presence of Pd–charcoal (10% Pd; 0·2 g.) until no more CO_2 was evolved. The solution was filtered and evaporated to dryness in a rotary evaporator.

The residual oil was dissolved in ether (8·0 ml.), and the required substance extracted into 2N-HCl (5·0 ml.). The acid solution was extracted six times with ether (5·0 ml. portions) until it was no longer fluorescent in ultraviolet light. The aqueous phase was then adjusted to pH 7·0 with 2N-NaOH and the oil which separated was extracted into ether (5·0 ml.). The whole extraction process was repeated twice more, and the final ethereal solution was shaken with 2N-HCl (2·0 ml.). The aqueous phase was evaporated to dryness *in vacuo* in the presence of solid NaOH.

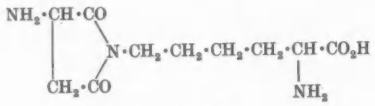
The pale-yellow solid remaining was triturated with acetone and gave a mass of white needles, which were collected, washed with acetone and dried. Yield 0·26 g. *L-Aminosuccinylisohexylamine hydrochloride* had m.p. 192–194° and partly sublimed just before melting. The sublimate was in the form of thin plates, but the starting material consisted of tiny needles (Found: C, 51·1; H, 8·1; N, 12·0; Cl, 15·3. $C_{10}H_{19}O_3N_2Cl$ requires: C, 51·2; H, 8·1; N, 12·0; Cl, 15·1%). The substance gave a single spot (R_F 0·78) on paper chromatography in butanol–acetic acid–water, and migrated towards the cathode as a single spot on paper electrophoresis at pH 7·0 (Fig. 2). The spots, developed with ninhydrin, were yellow–orange. On treatment at room temperature with 0·3N-$Ba(OH)_2$ for 0·5 hr., the substance yielded two products which were separated by electrophoresis on paper at pH 2·3 and corresponded to the α- and β-*iso*hexylamides of aspartic acid (Swallow *et al.* 1958). The β-isomer greatly predominated.

RESULTS

Cyclization of ε-aspartyl-lysines to ε-(aminosuccinyl)-lysine

Cyclization of ε-aspartyl-lysines in acid solution. ε-(α-L-Aspartyl)-L-lysine and ε-(β-L-aspartyl)-L-lysine behaved as neutral compounds during electrophoresis on paper at pH 7 (Fig. 2*A*, spot 1). After treatment with 11N-hydrochloric acid at 80° for 30 min. both products contained similar small amounts of free lysine and aspartic acid and a significant quantity of a basic substance which migrated, at pH 7, about half as fast as histidine towards the cathode (Fig. 2*A*, spot 2). After 10 hr. under the same conditions only a small amount of aspartyl-lysine remained and the intensity of the spots obtained with ninhydrin suggested that the major component of the mixture was the slow-moving base (spot 2). The relative intensities of the spots changed only very slowly when treatment with acid was prolonged further, but a gradual increase in the amounts of lysine and aspartic acid was observed (Table 1).

It seemed likely that the slow-moving base was ε-(aminosuccinyl)-lysine (I), whose formation from the neutral ε-aspartyl-lysines involved the masking of a carboxyl group. This view was confirmed when the substance was separated from the other products of the reaction and some of its properties were studied.



(I)

Separation of ε-(aminosuccinyl)-lysine. Two methods were used for the separation, on a preparative scale, of the products obtained when ε-aspartyl-lysines were treated with 11N-HCl at 80° for 24 hr. and the HCl was then removed *in vacuo*. The simplest procedure was electrophoresis in the Spinco continuous paper-electrophoresis apparatus, the buffer being pyridine acetate, pH 5 (see Experimental). Under the conditions used both ε-aspartyl-lysines were collected from drip points 9 to 11, ε-(aminosuccinyl)-lysine from 18 to 24, and lysine from 26 to 30. Aspartic acid migrated off the paper into the buffer circulating over the anode. The appropriate fractions were combined and the solutions freeze-dried. The residue of ε-(aminosuccinyl)-lysine was a colourless hygroscopic solid.

The second procedure involved removal of free aspartic acid by adsorption on a column of Dowex 1 × 10 (acetate form) and chromatography of the combined neutral and basic material on Dowex 50 × 4 in HCl (Hirs *et al.* 1954). The conditions used were very similar to those described below for the separation of ε-(aminosuccinyl)-lysine from hydrolysates of bacitracin (see Fig. 1). The

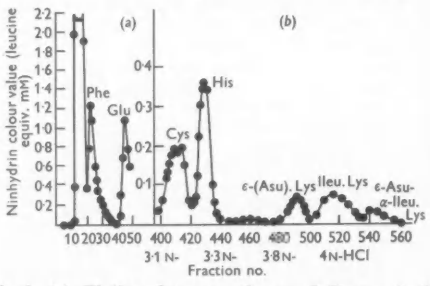


Fig. 1. (*a*) Elution from a column of Dowex 1 × 10 (25 cm. × 1·2 cm.) of a partial hydrolysate of bacitracin with 0·5N-acetic acid. Load, 250 mg.; flow rate, 4 ml./hr. Fractions each 1·0 ml. (*b*) Elution from a column of Dowex 50 × 4 (120 cm. × 2·0 cm.) of material corresponding to the first peak from (*a*). Eluent 1·0–4N-HCl; load, about 350 mg.; flow rate, 24 ml./hr.; fractions each 6·0 ml. The double peak in the cystine position indicates the presence of *meso*- and D- or L-isomers (cf. Hirs *et al.* 1954).

Table 1. *Hydrolysis under various conditions of ε-(α-aspartyl)-lysine, ε-(aminosuccinyl)-lysine and α-(α-aspartyl)-lysine*

The number of crosses indicates the approximate strength of amino acid or peptide as estimated visually from the intensities of the spots developed with ninhydrin after paper electrophoresis. The figures in parentheses show the approximate mol. prop. (%) of the components of the hydrolysate (see Experimental). Differences between the visual and numerical results are to be attributed to differences in ninhydrin colour yields. ε- and α-(Aminosuccinyl)-lysine are abbreviated to ε- and α-(Asu).Lys. α-(β-Asp).Lys and α-(α-Asp).Lys are not distinguished from each other.

	Conditions of hydrolysis			Composition of hydrolysate				
Substance	Reagent	Time (hr.)	Temp.	Asp	ε-(β-Asp).Lys	ε-(α-Asp).Lys	ε-(Asu).Lys	Lys
ε-(α-Asp).Lys		24	20°	.	.	+ + + +	+ +	.
		0·5	80	+	+	+ + +	+ + +	+
		2	80	+	+	+ +	+ + + +	+
	11 N-HCl	4	80	+	+	+	+ + + +	+
		24	80	+ + (9)	+ (3)	+ (0·7)	+ + + + (77)	+ + (10)
		70	80	+ +	+	+	+ + + +	+ +
		105	80	+ + +	+	+	+ + +	+ + +
		3	80	.	+	+ + + +	±	.
	0·1 N-HCl	24	80	+ +	+	+ + + +	+ +	+ +
		72	80	+ + + +	+	+ + +	+ +	+ + + +
	0·1 N-HCl	24	100	+ + + +	+	+	+ +	+ + + +
	6 N-HCl	24	105	+ + + +	.	.	.	+ + + +
		1	100	+	+	+ + + +	+	+
	0·25 N-Acetic acid	8	100	+ + +	+ + +	+ + +	+ + +	+ + +
		24	100	+ + + + (37)	+ + + (10)	+ (3)	+ + + (15)	+ + + + (35)
ε-(Asu).Lys		4	80	.	+	+	+ + + +	.
	11 N-HCl	24	80	+ (4·8)	+ (2)	+ (0·8)	+ + + + (90)	+ (3·5)
		43	80	+ +	+	+	+ + + +	+ +
	0·1 N-HCl	24	80	± (1·6)	+ + (6·3)	+ (1·6)	+ + + + (84)	± (1·6)
	0·3 N-$Ba(OH)_2$	0·5	18	.	+ + +	+	.	.
				α-(β-Asp).Lys plus α-(α-Asp).Lys			α-(Asu).Lys	
α-(α-Asp).Lys		0·5	80	±	+ + + +		+	±
		5	80	+ + +	+ + +		+ + +	+ + +
	11 N-HCl	24	80	+ + + +	+ +		+	+ + + +
		43	80	+ + + +	+		±	+ + + +

aspartyl-lysine remaining in the preparation emerged from the column of Dowex 50 as a small band immediately preceding lysine and the ε-(aminosuccinyl)-lysine as a relatively large band closely following lysine. The molecular ratio of ε-(aminosuccinyl)-lysine to lysine (calculated from ninhydrin colour densities) was 4 to 1. The fractions containing the ε-(aminosuccinyl)-lysine were evaporated *in vacuo* to yield a colourless hygroscopic glass.

Preparations of ε-(aminosuccinyl)-lysine obtained by these procedures appeared to be almost homogeneous when subjected to ionophoresis on paper at pH 2·3, 5 or 7. Only trace amounts of one other component, which appeared to be ε-aspartyl-lysine, could be detected with ninhydrin. However, attempts to obtain the compound in crystalline form, as a free base, a monohydrochloride, a picrate or a copper complex, were unsuccessful.

Properties of ε-(aminosuccinyl)-lysine. The infrared spectrum of ε-(aminosuccinyl)-lysine, unlike the spectra of the ε-aspartyl-lysines, contained a medium band at 1785 cm.$^{-1}$ and a strong band at 1705 cm.$^{-1}$. These bands could be assigned to the carbonyl groups of a succinimide ring (Randall, Fowler, Fuson & Dangl, 1949). The spectra of the ε-aspartyl-lysines showed a strong band at 3300 cm.$^{-1}$ which could be assigned to the NH stretching mode of the peptides. This band was absent from the spectrum of ε-(aminosuccinyl)-lysine.

On paper chromatograms run with butanol–acetic acid–water ε-(aminosuccinyl)-lysine was not separated from lysine or from the two ε-aspartyl-lysines. These substances were readily distinguished, however, by electrophoresis on paper in collidine acetate (pH 7·0), pyridine acetate (pH 5·0) or aqueous acetic acid (pH 2·3) (Fig. 2*A–C*).

The fact that ε-(aminosuccinyl)-lysine migrated towards the cathode much more rapidly at pH 5 than at pH 7 (Fig. 2*A* and *B*) indicated that one of its functional groups ionized in this pH region. Electrometric titration (Fig. 3) showed that it contained three ionizable groups whose pK_a values were 2·5, 6·0 and 9·5 respectively. The pK_a of less than 2·5 could be assigned to a carboxyl group, that of 6·0 to the amino group of an aminosuccinimide ring and that of 9·5 to the α-amino group of a lysine residue.

When the titration was carried to a pH higher than 10 an irreversible change began. Back-titration of a solution which had been kept at pH 11 for 30 min. showed that the group with pK_a 6·0 had disappeared. The resulting curve (Fig. 3) suggested that hydrolysis had occurred with the formation of a mixture of ε-(α-aspartyl)- and ε-(β-aspartyl)-lysine (cf. Swallow *et al.* 1958). The product obtained when hydrolysis was allowed to occur in 0·3 N-barium hydroxide at 20° for 30 min. gave a single spot in a position corresponding to both ε-aspartyl-lysines when subjected to electro-

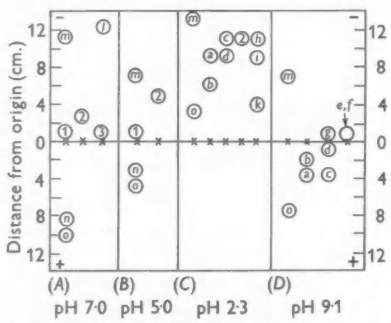


Fig. 2. Electrophoresis on paper (14 v/cm. for 2·5 hr.) in (*A*) collidine acetate (pH 7·0), (*B*) pyridine acetate (pH 5·0), (*C*) aqueous acetic acid (pH 2·3), and (*D*) sodium borate (pH 9·1). Letters refer to single substances and numbers to groups of substances. In systems *A*, *B* and *C* glucose was carried 0·5 cm. towards the cathode by electroendosmosis, and in system *D* DNP-ethanolamine was carried 1·6 cm. towards the cathode. Group 1: *a*, ε-(α-aspartyl)-lysine; *b*, ε-(β-aspartyl)-lysine; *c*, α-(α-aspartyl)-lysine; *d*, α-(β-aspartyl)-lysine. Group 2: *e*, ε-(aminosuccinyl)-lysine (synthetic); *f*, ε-(aminosuccinyl)-lysine (from bacitracin A); *g*, α-(aminosuccinyl)-lysine; *h*, *N*-aspartoyl*iso*hexylamine. Group 3: *i*, α-*iso*hexylamide of aspartic acid; *k*, β-*iso*hexylamide of aspartic acid. *l*, *iso*hexylamine; *m*, lysine; *n*, glutamic acid; *o*, aspartic acid.

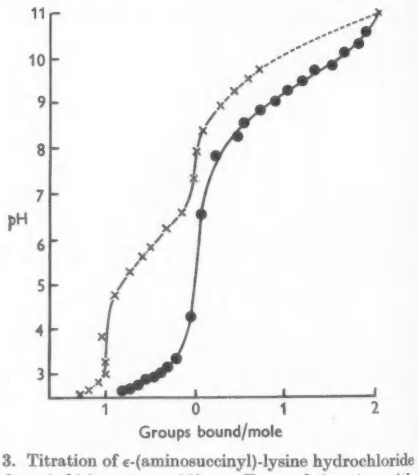


Fig. 3. Titration of ε-(aminosuccinyl)-lysine hydrochloride (1·6 mg./ml.) in water at 20°. ×, Forward titration with 0·1 N-NaOH; ●, back titration with 0·1 N-HCl of a solution kept at pH 11 for 30 min.

phoresis on paper at pH 7 or at pH 5 (Fig. 2*A*, *B*, spot 1). After electrophoresis at pH 2·3 it showed a major spot in the position corresponding to ε-(β-aspartyl)-lysine and a minor spot in that corresponding to ε-(α-aspartyl)-lysine (Fig. 2*C*, spots *b* and *a*). In contrast with its lability in alkaline solution, ε-(aminosuccinyl)-lysine was remarkably resistant to hydrolysis in 11N-hydrochloric acid at 80° (Table 1).

The DNP derivative of ε-(aminosuccinyl)-lysine yielded DNP-aspartic acid and α-DNP-lysine on hydrolysis and also an ether-soluble product which remained at the origin on paper chromatograms (Blackburn & Lowther, 1951) and showed a bright-green fluorescence in ultraviolet light (365 mμ). A similar product was reported by Lockhart & Abraham (1956) to be formed on reaction of the 'Asp.Lys' from bacitracin A with 1-fluoro-2:4-dinitrobenzene.

Interconversion of ε-(α-aspartyl)- and ε-(β-aspartyl)-lysine

Electrophoresis on paper at pH 2·3 showed that ε-(α-aspartyl)-lysine was converted largely into ε-(aminosuccinyl)-lysine by treatment with 11N-hydrochloric acid for 10 hr. at 80°. In addition to this product and some free lysine and aspartic acid, however, a small spot corresponding to the original peptide was discernible, and also a second spot, of similar intensity, corresponding to ε-(β-aspartyl)-lysine. The concentrations of the two ε-aspartyl-lysines then appeared to undergo relatively little change when treatment with acid was prolonged to 70 hr. (Table 1). Similar results were obtained with ε-(β-aspartyl)-lysine, which was converted into a mixture containing small amounts of both aspartyl-lysines, as well as larger amounts of lysine, aspartic acid, and ε-(aminosuccinyl)-lysine, on prolonged treatment with 11N-hydrochloric acid at 80°. A similar conversion of ε-(α-aspartyl)-lysine into the β-aspartyl isomer was observed in 0·25N-acetic acid at 100°. In this case, however, the intensity of the spot due to the β-isomer was considerably greater than that due to the α-isomer after 24 hr. (Table 1).

Cyclization of ε-(α-L-aspartyl)-L-lysine under other conditions

In 0·1N-hydrochloric acid at 80° both the rate of hydrolysis of ε-(α-L-aspartyl)-L-lysine to aspartic acid and lysine and its cyclization to the succinimide were much slower than in 11N-acid. After 72 hr. a considerable proportion of the original peptide remained unchanged (Table 1). However, the reduction in the rate of hydrolysis was less than the reduction in the rate of imide formation, and the amounts of lysine and aspartic acid formed in the early stages of the reaction were relatively greater than the amount of imide. After electrophoresis on paper (pH 2·3) of the reaction products at times varying from 1 to 24 hr. the spots corresponding to imide, lysine and aspartic acid were all of comparable intensity. The imide itself underwent very little hydrolysis during 24 hr. in 0·1N-hydrochloric acid at 80°, but it yielded a small amount of the β-aspartyl peptide and a trace of α-isomer.

In 0·1N- or N-hydrochloric acid at 100° the rate of formation of aspartic acid and lysine was large compared with the rate of accumulation of imide. This probably implies that the ring-opening of the imide is associated with a higher energy of activation than is the hydrolysis of the dipeptide. Although the imide is relatively stable in 6N-hydrochloric acid at 80° it is completely hydrolysed in 24 hr. at 105°. In 11N-hydrochloric acid at 20° ε-(α-L-aspartyl)-L-lysine yielded a significant amount of imide in 24 hr. but no detectable quantity of lysine or aspartic acid.

ε-(Aminosuccinyl)-lysine from bacitracin A

The compound detected by Lockhart & Abraham (1956) in hydrolysates of bacitracin A, and provisionally described as Asp.Lys, was separated by ion-exchange chromatography from other products formed when bacitracin was treated with 11N-hydrochloric acid at 80°. Free aspartic and glutamic acids were first removed by adsorption on a column of Dowex 1 × 10 acetate. Under the conditions used, phenylalanine was also removed at this stage, since it emerged from the column as a sharp peak immediately following a mixture of the basic and remaining neutral products (Fig. 1*a*). The latter was chromatographed on Dowex 50 × 4 in hydrochloric acid whose concentration was raised from 1·0 to 4·0N (Fig. 1*b*). The compound 'Asp.Lys' emerged from the column after histidine (fractions 480–500). It was followed by Ileu.Lys and then by the compound described provisionally by Lockhart & Abraham (1956) as Asp(Ileu).Lys.

Evaporation of fractions 480–495 *in vacuo* yielded an amorphous residue. This product migrated towards the cathode at the same rate as ε-(aminosuccinyl)-lysine (obtained from ε-aspartyl-lysine) when subjected to electrophoresis on paper at pH 7. The product also migrated at the same rate as ε-(aminosuccinyl)-lysine on electrophoresis at pH 5·0 and 2·3 (Fig. 2, spot 2), and was not separated from ε-(aminosuccinyl)-lysine by chromatography on paper in butanol–acetic acid–water. On treatment with 0·3N-barium hydroxide at room temperature for 1 hr. it was converted entirely into material which showed no net charge at pH 7. Electrophoresis on paper at pH 2·3 showed that this material consisted of two ninhydrin-positive components, the major component migrating towards the cathode at the same rate as ε-(β-aspartyl)-lysine and the minor component at

the same rate as ε-(α-aspartyl)-lysine. In this behaviour, also, the compound from bacitracin A was indistinguishable from ε-(aminosuccinyl)-lysine.

Licheniformins A, B and C, which, like bacitracin, are produced by strains of *Bacillus licheniformis* (Callow & Work, 1952), yielded no detectable amount of ε-(aminosuccinyl)-lysine in 11N-hydrochloric acid at 80°.

ε-(*Aminosuccinyl*)-*lysine from cell walls of* Lactobacillus brevis

Cell walls of *Lactobacillus brevis* were treated with 11N-hydrochloric acid at 80° for 43 hr. When the product was subjected to electrophoresis on paper at pH 7, a neutral spot, two acidic spots and three basic spots were observed. A major acidic spot corresponded to glutamic acid and a minor one to aspartic acid. Two of the basic spots corresponded to lysine and glucosamine respectively, the former migrating slightly faster than the latter. The third basic spot, which was the most intense of the three, migrated to the same position as ε-(aminosuccinyl)-lysine (Fig. 2*A*).

The material corresponding to the third basic spot was eluted from the paper after electrophoresis at pH 7 on a larger scale. This material migrated towards the cathode at the same rate as ε-(aminosuccinyl)-lysine on electrophoresis at pH 5·0 and 2·3. On hydrolysis with 6N-hydrochloric acid at 110° for 24 hr. it yielded equivalent amounts of lysine and aspartic acid. On treatment with 0·3N-barium hydroxide at 20° for 1 hr. it yielded two products which were separated by electrophoresis on paper at pH 2·3. The major product migrated at the same rate as ε-(β-aspartyl)-lysine and the minor one at the same rate as ε-(α-aspartyl)-lysine (Fig. 2*C*, spots *a* and *b*).

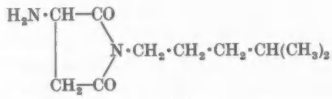
These results left little doubt that the third basic product from the cell walls of *Lactobacillus brevis* was ε-(aminosuccinyl)-lysine. The possibility that it was α-(aminosuccinyl)-lysine was later eliminated when it was shown that this substance was much less stable to acid than the ε-isomer, and that ε-(β-aspartyl)-lysine was readily distinguishable from α-(β-aspartyl)-lysine by electrophoresis on paper (Fig. 2*C* and *D*).

No ε-(aminosuccinyl)-lysine was detected in the cell walls of a strain of *Bacillus licheniformis* which produces bacitracin.

Cyclization of α- *and* β-iso*hexylamides of aspartic acid*

When either the α- or β-*iso*hexylamide of aspartic acid (Swallow *et al.* 1958) was treated with 11N-hydrochloric acid at 80° for 20 hr. some free aspartic acid and *iso*hexylamine were formed and also a considerable amount of a basic product which showed a mobility similar to that of ε-(aminosuccinyl)-lysine on paper electrophoresis at pH 7 (Fig. 2*A*). This product was revealed as a yellow–orange spot when the paper was sprayed with ninhydrin. It was isolated as a crystalline hydrochloride (m.p. 192–194°) by extraction into ether from an aqueous solution at pH 7, re-extraction into 2N-hydrochloric acid, removal of the acid *in vacuo*, and trituration of the remaining solid with acetone. When treated with 0·3N-barium hydroxide at 20° it was converted into two compounds which were indistinguishable from the α- and β-*iso*-hexylamides of aspartic acid respectively on electrophoresis at pH 2·3 (Fig. 2*C*). The relative intensities of the spots showed that the yield of the β-isomer was considerably higher than that of the α-isomer.

These properties indicated that the basic product was aminosuccinyl*iso*hexylamine (II).



(II)

Structure (II) was supported by the fact that the product was indistinguishable in its electrophoretic mobility at pH 7, and its behaviour in alkaline solution, from a compound, $C_{10}H_{18}O_2N_2$, synthesized from benzyloxycarbonyl-L-aspartic anhydride and *iso*hexylamine (see Experimental). The compound $C_{10}H_{18}O_2N_2$ showed an infrared spectrum containing two strong bands at 1785 and 1710 cm.$^{-1}$ respectively which could be attributed to the carbonyl groups of a succinimide ring (Randall *et al.* 1949).

Cyclization of α-(α-L-*aspartyl*)-L-*lysine and* α-(β-L-*aspartyl*)-L-*lysine*

α-(α-L-Aspartyl)-L-lysine and α-(β-L-aspartyl)-L-lysine behaved as neutral compounds on paper electrophoresis at pH 7, but were separated by electrophoresis at pH 2·3 or 9·1. The α-(α-aspartyl) isomer was distinguished from ε-(α-aspartyl)-lysine by its greater mobility (towards the cathode) at pH 2·3, and the α-(β-aspartyl) isomer was distinguished from ε-(α-aspartyl)-lysine by its smaller mobility (towards the anode) at pH 9·2 (Fig. 2*C* and *D*).

When α-(α-L-aspartyl)- and α-(β-L-aspartyl)-L-lysine were treated with 11N-hydrochloric acid at 80° a basic product was formed, in addition to free lysine and aspartic acid, which showed the same mobility as ε-(aminosuccinyl)-lysine on paper electrophoresis at pH 7 (Fig. 2*A*). This product was undoubtedly α-(aminosuccinyl)-lysine. It was considerably less stable to acid, however, than the

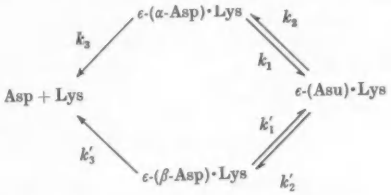
ε-isomer, reaching a maximal and relatively low concentration after 5 hr. and thereafter rapidly diminishing. After 48 hr. only a trace of the compound remained and hydrolysis to aspartic acid and lysine approached completion.

DISCUSSION

Cherbuliez & Chambers (1924) reported that benzamidosuccinimide could be obtained by the action of heat on *N*-benzoylasparagine. Benzyloxycarbonylaminosuccinimide was later prepared by the saponification of *N*-benzyloxycarbonyl-L-asparagine methyl ester under mild conditions and yielded L-aminosuccinimide itself on removal of the benzyloxycarbonyl group (Sondheimer & Holley, 1954*a*, *b*). The cyclization in alkaline solution was assumed to be initiated by the removal of a proton from the amide nitrogen. Battersby & Robinson (1955) showed that the esters of *N*-benzoyl-α-aspartyl peptides and the corresponding β-aspartyl peptides were partly converted into derivatives of aminosuccinimide on treatment with less than one equivalent of dilute sodium hydroxide. Treatment of the imide with an excess of alkali resulted in ring-opening and the formation of a mixture of *N*-benzoylaspartyl peptides, the β-isomer predominating.

The ε-(aminosuccinyl)-lysine encountered here has not been obtained in a crystalline form, but its structure appears to have been clearly established. The pK_a of its first amino group (6·0) is very close to that reported for the amino group of aminosuccinimide (5·9). It is formed under conditions similar to those under which the well-characterized aminosuccinyl*iso*hexylamine is produced from the *iso*hexylamides of aspartic acid, and its infrared spectrum shows two bands which can be attributed to the CO groups of the succinimide ring. Like the derivatives of aminosuccinimide described previously, it is readily converted by dilute alkali into a mixture of α- and β-aspartyl peptides. The predominance of the β-isomer is to be expected, since the reaction is presumably initiated by the nucleophilic attack of a hydroxyl ion on the two carbonyl groups of the ring, and the carbon derived from the α-carboxyl group of aspartic acid will have the greater fractional positive charge.

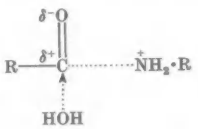
John & Young (1954) reported that α-L-aspartyl-L-valine was partly converted into β-L-aspartyl-L-valine when heated in aqueous solution at 100° for 6 hr., but they did not detect any cyclic intermediate. Detection of the formation of ε-(aminosuccinyl)-lysine [ε-(Asu).Lys] was facilitated by the characteristic behaviour of this compound on electrophoresis and by its great stability in concentrated hydrochloric acid. When ε-(α-aspartyl)-lysine [ε-(α-Asp).Lys] or its isomer [ε-(β-Asp).Lys] is heated in acid solution the following reactions occur, the velocity constants being represented by k:



The intensities of spots obtained after electrophoresis on paper indicated that the values of k_1, k_2 and k_3 were of the same order as those of k'_1, k'_2 and k'_3 respectively, although k'_2 was somewhat greater than k_2. After 10 hr. in 11 N-hydrochloric acid ε-(α-Asp).Lys and ε-(β-Asp).Lys reached almost stationary concentrations which were very low in comparison with that of ε-(Asu).Lys. Thus $k_2+k'_2 \ll (k_1+k'_1)+(k_3+k'_3)$. It also appeared that $k_3 \ll k_1$ and $k'_3 \ll k'_1$, so that $(k_2+k'_2) \ll (k_1+k'_1)$. In other words, the rate of cyclization under these conditions was much greater than the rate of ring-opening.

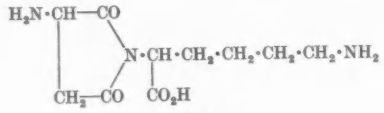
In 0·1 N-hydrochloric acid at 80° the values of k_1, k_2 and k_3 were all lower than the corresponding values in 11 N-acid. However, the quotient k_3/k_1 was considerably larger in the dilute acid than in the concentrated, and in the dilute acid hydrolysis competed with imide formation on similar terms.

These results may be explicable in terms of changes in the activity of water. The hydrolysis of amides in acid solution is believed to involve protonization of the amide nitrogen and separation of this nitrogen from the cationoid carbon with concomitant attack on the latter by the oxygen of water (Meloche & Laidler, 1951; Bender & Ginger, 1955):



The rate of hydrolysis rises to a maximum as the concentration of acid is increased (to 6 N-HCl with formamide) and then declines. The rise is attributed to the conversion of the amide into the conjugate acid, and the subsequent decline to a fall in the activity of water. In the cyclization leading to aminosuccinimides a molecule of water is eliminated and the activity of water in the solution may play a dominant role. Once the imide is formed the resistance of its very weakly basic nitrogen to the addition of a proton may account for its high stability in acid solution.

When α-(α-aspartyl)-lysine was kept at 80° in 11N-hydrochloric acid the values of k_1 and k_3 appeared to be similar to those for the corresponding ε-isomer, but the value of k_2+k_2' was much larger. The reason for the lower stability of the imide (III)



(III)

from α-(α-aspartyl)-lysine is not clear, since the proximity of the carboxyl group to the ring nitrogen should make the latter more difficult to protonate. Possibly steric factors are involved, or the carboxyl group itself plays a part in ring-opening. Partridge & Davis (1950) showed that there was a preferential liberation of aspartic acid from certain proteins on hydrolysis with weak acids and suggested that the β-carboxyl group of aspartic acid was acting, under these conditions, as a proton donor. The α-amino and carboxyl groups of ε-(aminosuccinyl)-lysine do not appear to be concerned in its resistance to ring-opening, because *N*-aspartoyl*iso*hexylamine shows a similar stability.

It follows from the results obtained here that a mixture of α- and β-aspartyl peptides may be formed on partial hydrolysis, in concentrated hydrochloric acid, of polypeptide chains containing the sequence Asp.Lys. Whether extensive inversion may occur under such conditions when aspartic acid is linked to other amino acids has not been established, but the finding of an α- or β-aspartyl peptide in acid hydrolysates must clearly be interpreted with caution.

The formation of ε-(aminosuccinyl)-lysine from bacitracin A confirms the conclusion of Lockhart & Abraham (1956) that one of the two aspartic acid residues in the antibiotic is linked to the ε-amino group of lysine. The product of hydrolysis of bacitracin A which was provisionally described as Asp(Ileu).Lys (Lockhart & Abraham, 1956) is doubtless ε-(aminosuccinyl)-α-isoleucyl-lysine. There is as yet no evidence that the aminosuccinimide ring occurs in bacitracin A itself, and it would be difficult to reconcile its presence with other evidence about this portion of the molecule (Lockhart & Abraham, 1954*a*; Hausmann, Weisiger & Craig, 1955). However, the formation of the ring and interconversion of the aspartyl-lysines make it impossible to determine, from the products of hydrolysis, whether the original sequence consists of an α- or β-aspartyl peptide.

The lysine residues in proteins normally have their ε-amino groups free, although a peptide in which an ε-amino group of a lysine residue is bound has recently been obtained from collagen (Mechanic & Levy, 1957). It is therefore of interest that the unusual ε-aspartyl-lysine sequence occurs in the cell walls of *Lactobacillus brevis*. This sequence is also present in the cell walls of *L. acidophilus*, *L. helveticus*, *L. fermenti*, *L. casei*, *L. delbrueckii*, and some strains of *Actinomyces bovis*, since Cummins & Harris (1956, 1958) have detected in acid hydrolysates of these walls a substance with similar properties to that shown here to be ε-(aminosuccinyl)-lysine. The sequence is probably responsible, too, for a substance with similar properties detected by Powell & Strange (1957) in peptides released from the cell walls of *Bacillus sphaericus* by lysozyme. Whether the ε-(Asp).Lys sequence in bacterial cell walls contains aspartic acid and lysine residues with the same optical configuration as the corresponding residues in bacitracin remains to be determined.

Extracellular polypeptides may well be synthesized near the cytoplasmic membrane of the bacterial cell and it seemed possible that the ε-aspartyl-lysine fragment of bacitracin A was produced by a mechanism which was also involved in the synthesis of a fragment of the cell wall. However, ε-(aminosuccinyl)-lysine has not been detected in hydrolysates of the cell wall of a strain of *Bacillus licheniformis* used for producing bacitracin. It has also not been detected in hydrolysates of the cell walls of two strains of *L. bifidus*, although the walls of these organisms contain lysine and aspartic acid residues (Cummins, Glendenning & Harris, 1957). Possibly in those bacterial cell walls which yield ε-(aminosuccinyl)-lysine on hydrolysis a lysine residue is the site of a branch in a peptide chain, as it is in bacitracin A.

SUMMARY

1. ε-(α-L-Aspartyl)-L-lysine and ε-(β-L-aspartyl)-L-lysine cyclize to ε-(L-aminosuccinyl)-L-lysine in 11N-hydrochloric acid at 80°. Cyclization occurs more rapidly than hydrolysis to lysine and aspartic acid. The α- and β-*iso*hexylamides of aspartic acid cyclize in a similar manner.

2. ε-(Aminosuccinyl)-lysine shows considerable stability to 11N-hydrochloric acid at 80°, but it is slowly hydrolysed to lysine and aspartic acid with the intermediate formation of the two ε-aspartyl-lysines. Both aspartyl-lysines reach only a relatively low stationary concentration, but that of the β-isomer is somewhat higher than that of the α-isomer.

3. ε-(Aminosuccinyl)-lysine is rapidly hydrolysed in cold dilute alkali to a mixture of α- and β-aspartyl-lysines in which the β-isomer greatly predominates.

4. α-(α-L-Aspartyl)-L-lysine and α-(β-L-aspartyl)-L-lysine cyclize to α-(L-aminosuccinyl)-L-lysine in 11N-hydrochloric acid at 80°, but this derivative of aminosuccinimide is much less stable than that formed from the corresponding ε-aspartyl-lysines.

5. ε-(Aminosuccinyl)-lysine is formed when bacitracin A is treated with 11N-hydrochloric acid at 80°. It is also formed during acid hydrolysis of the cell walls of certain strains of lactobacilli. The cell walls of other strains of lactobacilli do not yield this compound, although they contain lysine and aspartic acid residues.

One of us (D.L.S.) is indebted to the Medical Research Council for a scholarship. Infrared spectra were determined by Dr F. B. Strauss. The analysis was by Weiler and Strauss.

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The Production of an Esterase Inhibitor from Schradan in the Fat Body of the Desert Locust

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The oxidative conversion of the systemic insecticides schradan (bisdimethylaminophosphonous anhydride) and dimefox (bisdimethylaminofluorophosphine oxide) into powerful anticholinesterases has been studied in mammalian liver homogenates (Davison, 1954, 1955; O'Brien, 1956, 1957; Fenwick, Barron & Watson, 1957), and shown to be dependent on the presence of Mg^{2+} or Ca^{2+} ions, nicotinamide and diphosphopyridine nucleotide. The microsome fraction of liver cells could effect the conversion if fortified with reduced diphosphopyridine nucleotide. O'Brien & Spencer (1953, 1955) reported that many intact insect tissues could produce the same schradan metabolite as did liver, but all attempts to make an active tissue homogenate were unsuccessful. Cockroach-gut homogenates fortified with nicotinamide, diphosphopyridine nucleotide and Mg^{2+} ions failed to convert schradan (O'Brien, 1957) and dimefox (Fenwick *et al.* 1957) into such compounds, and it was suggested that a different mechanism might be involved.

An investigation of the conversion of schradan by the tissues of the desert locust was undertaken in order to determine whether there is a fundamental difference of mechanism between mammals and insects. The choice of insect was governed by its size, availability and economic importance.

MATERIALS AND METHODS

Schradan, diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN) and flavine mononucleotide (FMN) were obtained from L. Light and Co. Ltd.; dimefox from Fisons Pest Control Ltd.; flavinadenine dinucleotide (FAD) from Sigma Chemical Co.; DPNH (reduced DPN) was prepared by the reduction of DPN with dithionite (Colowick & Kaplan, 1955), and TPNH (reduced TPN) by the enzymic method of Nason & Evans (1953). *p*-Nitrophenyl acetate was synthesized by the method of Kaufmann (1909).

Fourth-instar nymphs of the desert locust (*Schistocerca gregaria* Forsk.) were provided at regular intervals by the Anti-Locust Research Centre, 1 Princes Gate, London, S.W. 7. The insect cage stood in a thermoregulated room, and was lit and heated internally by an electric-light bulb which was automatically switched on at 8 a.m. and off at midnight. The mean day temperature inside the cage was 35°, falling to 25° at night. A tray of water stood beneath the wire-gauze floor of the cage, and the humidity was further influenced by a jar of fresh or cold-stored green grass and a dish of drinking water in the cage. The grass diet was supplemented by a mixture of grass meal, bran, dried whole milk and dried autolysed yeast (The Distillers Co. Ltd.) in the proportions 2:2:2:1 (by vol.).

The tissues were removed from the decapitated insects and placed in an ice-cooled dry beaker. Air sacs and tracheae were generally not separated from the fat body after it was found that their presence did not affect schradan conversion. Testes were removed before the fat body, as otherwise they tended to become incorporated in it. The unwashed tissue was homogenized with a glass Potter–Elvehjem tube and pestle. Fat-body tissue is very soft, and five or six up-and-down movements of the pestle were sufficient to release the fat from the cells.

Esterase determination. The principle of the method of Huggins & Lapides (1947) was used, the rate of liberation of *p*-nitrophenol from *p*-nitrophenyl acetate being measured. The substrate could be kept for up to a month at −15° as a 50 mM-solution in dry methanol. For each series of estimations it was diluted (1/25) with water, and used within 1 hr. Esterase preparation (1 ml.) was added to 3 ml. of 0·01 M-phosphate buffer, pH 7·4 (Umbreit, Burris & Stauffer, 1949), in a 1 cm. diam. spectrophotometer tube. A blank contained water instead of esterase preparation. At zero time, 1 ml. of substrate solution was added to the first tube and its optical density at 400 mμ immediately measured with a Unicam SP 400 instrument. Subsequent tubes were treated likewise at intervals of 1 min., and the tubes allowed to stand at room temperature. At 15 min. a series of final readings was started, and the change of optical density of each tube was referred to a *p*-nitrophenol standard curve. The blank (spontaneous hydrolysis) value was subtracted from each. The activity, expressed as μmoles of substrate hydrolysed/hr./ml. of enzyme, was directly proportional to enzyme concentration up to an optical-density change of 0·33.

Schradan conversion by intact tissue. Batches of fat-body tissue (30 mg. wet wt.) were incubated in air for 30 min. at 37° in 2 ml. of 'insect Ringer' solution (Thomsen, 1952), buffered with 10% (v/v) of 0·1 M-phosphate buffer, pH 7·4, in the presence of schradan at concentrations of 25, 50, 100 and 500 μM. The tissue was then homogenized in the medium, water was added to give a total of 1 ml./3–6 mg. of tissue, and the suspension was centrifuged for 2 min. at 600 *g*. A volume (1 ml.) of supernatant was taken for esterase measurement after removal of the upper layer of fat. Converting power was expressed as the mean of the four values of log (100/*b*)/mg. dry wt./concn. of schradan (M) which were so obtained, where *b* is the percentage of the initial esterase which remained.

Dry weight and fat content were obtained by homogenizing a weighed sample of tissue in a small volume of water and shaking with ether. After a brief centrifuging, a portion of the ether extract was dried at room temperature and weighed. The aqueous suspension, freed of the ether layer, was dried at 110° and weighed.

Schradan conversion by homogenates. A fat-body homogenate in water was incubated for 3 hr. at 37° to reduce its Schradan-converting power. Schradan concentrations up to mM had no significant effect on its esterase activity. mM-Schradan was therefore usually used in subsequent conversion experiments, and schradan was omitted from controls. The components were placed in 6 in. × 1 in. boiling tubes shaken in a water bath at 37° and open to the air. A typical reaction mixture consisted of 0·2 ml. of fat-body suspension, 0·2 ml. of inhibitor or activator dissolved in 0·25 M-sucrose and 0·1 ml. of 5 mM-schradan in 0·1 M-phosphate buffer, pH 7·4. At the end of the incubation period (usually 30 min.) water was added to stop the reaction by dilution and cooling, and to give a convenient concentration of esterase, which was then estimated.

Centrifugal fractionation. A homogenate of 150–200 mg. wet wt. of fat body/ml. of 0·25 M-sucrose solution was centrifuged for 2 min. in a bench centrifuge (about 600 *g*), and the supernatant was decanted after removal of the upper layer of fat by suction. This did not reduce the schradan-converting activity of the suspension. The supernatant was then centrifuged at 0° for a further 30 min. at 18 000 *g*. The resulting clear supernatant was carefully pipetted off and replaced by an equal volume of sucrose solution in which the sedimented particles were resuspended.

RESULTS

Intact tissue

The intact gut and fat body of locusts of various ages of the fifth-instar and adult forms were tested for schradan conversion, with endogenous esterase as an indicator of inhibitor produced. The gut (slit longitudinally and cleaned of food) showed no activity with mM-schradan at 37°, pH 7·4, but fat body possessed definite but erratic activity. This was found to vary with the age of the insect, although males and females of the same age did not differ appreciably. A systematic investigation of the fat body of the fifth-instar and adult locusts

(fourth-instar and younger forms are too small to constitute a convenient source of material) showed that schradan-converting power began to decline sharply 2 days before the final moult, and rose slightly during the first 3 weeks of adult life (Table 1). It was noticed also that the esterase content declined during the fifth-instar and remained low in the adult. Fat body from fourth–sixth day fifth-instar locusts was therefore used in subsequent experiments.

Fat-body homogenates

A homogenate of fat body in 0·25 M-sucrose was found to be surprisingly active in schradan conversion. mM-Schradan incubated for 30 min. at 37° with a 5 % (wet wt.) suspension of fat body, buffered with phosphate buffer, pH 7·4, caused a marked inhibition of the endogenous esterase.

Rate of reaction. When time of incubation was plotted against log (100/b) (Aldridge, 1950), an S-shaped curve was obtained (Fig. 1), probably owing to the initial gradual increase of the inhibitor concentration and the final decline of activity of the homogenate. However, log (100/b) gave a better approximation to a straight line than percentage inhibition (i.e. $100-b$) when plotted against time, and so was used as an estimate of the converting power of a fat-body preparation.

Buffers. Since O'Brien (1957) stated that schradan conversion by whole cockroach gut is inhibited by phosphate buffer, glycylglycine and 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffers at pH 7·4 (Colowick & Kaplan, 1955) were substituted for phosphate, but with no advantage.

Metals. $CaCl_2$ and $MgSO_4$ both enhanced the converting power of fresh sucrose homogenates to about the same extent (e.g. in a typical experiment, mM-$CaCl_2$ soln. increased the inhibition of esterase from 25 to 40 %). The optimum effect was achieved with $CaCl_2$ at concentrations of 0·8 mM or higher. $BaCl_2$ and $MnCl_2$ had a small activating effect. $ZnCl_2$ (mM), $CuSO_4$ (0·1 mM), $FeCl_3$ (mM), $CoCl_2$, NaCl and Na_2SO_4 (all 10 mM) had none.

Cofactors. The presence of 10 mM-nicotinamide in the homogenizing medium did not improve converting power. The diphosphopyridine nucleotidase activity was measured and found to be less than 5 % of that of rat liver (/g. fresh wt.), which probably accounts for the lack of effect with nicotinamide.

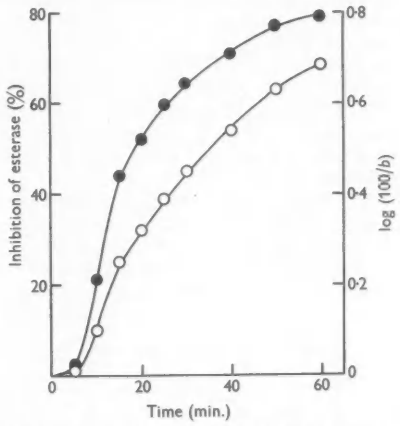


Fig. 1. Conversion of schradan by fat-body homogenate. Fat-body suspension, 60 mg./ml. in 0·25 M-sucrose and 10 mM-$MgSO_4$, was incubated with mM-schradan, pH 7·4, at 37°. Samples were removed at intervals, and diluted with cold water and their esterase activities measured. ●, Percentage inhibition of esterase; ○, log (100/b), where b = percentage of initial esterase remaining.

Table 1. *Variation with age of schradan-converting power of intact fat body*

In each case the tissue from at least four locusts (equal numbers of males and females) was pooled. Details of procedure are given in the Materials and Methods section. The final moult generally occurred on the eighth day of the fifth instar.

Age (days)	Fat body/locust (mg. wet wt.)	Fat (%)	Dry wt. (%)	Esterase activity*	Schradan conversion†
Fifth instar					
4	50	28	23	0·49	1·6
5	56	24	23	0·36	1·6
6	71	32	19	0·31	1·3
7	75	33	14	0·21	0·05
8	50	27	11	0·23	0·06
Adult					
7	89	59	15	0·27	0·02
11	125	64	16	0·17	0·17
14	219	64	14	0·17	0·21
19	131	55	11	0·33	0·30

* μmoles of *p*-nitrophenyl acetate hydrolysed/15 min./mg. dry wt.

† Mean of four values of log (100/b)/M-concn. of schradan/mg. dry wt.; b = percentage of initial esterase remaining.

DPN and TPN (mM) were added to the homogenate in sucrose with or without nicotinamide, but had no effect.

Dialysis for 16 hr. at 2° against isotonic sucrose soln. resulted in complete loss of activity, which was not restored by DPN, TPN, DPNH, TPNH or by the clear supernatant obtained by centrifuging for 1 hr. at 18 000 *g*.

Fractionated homogenate. Both the supernatant and particulate fractions obtained by centrifuging contained enough esterase for a schradan-conversion test to be carried out. Neither was active alone, but when they were recombined full activity was restored.

Function of the supernatant. DPNH was prepared chemically and shown to fortify rat-liver microsomes in the conversion of schradan, but it would not replace the supernatant. Nor would a system generating DPNH, consisting of an extract of dried baker's yeast (containing alcohol dehydrogenase), ethanol and DPN.

A TPNH-producing system, comprising an extract of acetone-dried pig heart (containing *iso*citric dehydrogenase), DL-*iso*citrate (0·01 M) and TPN, was next added to a fat-body particle preparation, which could then convert schradan. The efficiency of conversion depended on the amount of TPN added, and reached a maximum with about 2 mM-TPN. TPNH was prepared with the same pig-heart enzyme, which was then destroyed by boiling in alkaline solution. Schradan conversion by particles was shown to depend on TPNH concentration, reaching an optimum equal to the rate with supernatant, at about mM (Fig. 2).

When TPN was added to the fat-body supernatant, an increase in optical density at 340 mμ occurred which appeared to be limited by the concentrations of endogenous substrates, for when *iso*citrate, glucose 6-phosphate or malate was added, it increased further. Dialysis removed the TPN-reducing activity and addition of these substrates restored it (Fig. 3). There was no increase in optical density at 340 mμ when DPN was added to the supernatant.

No conversion was observed by particles in the presence of mM-hydrogen peroxide or 2 mM-DL-ascorbic acid.

Endogenous-reduced triphosphopyridine nucleotide. When the supernatant was replaced by the TPN-reducing system, TPN had to be added to an

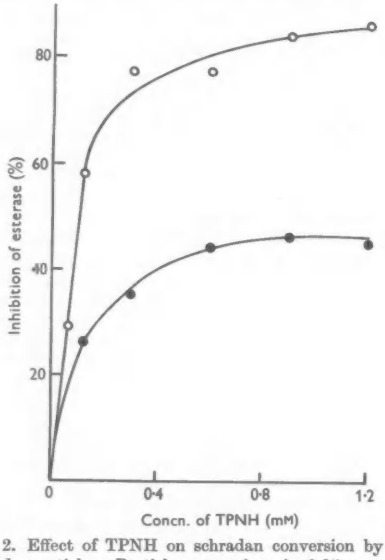


Fig. 2. Effect of TPNH on schradan conversion by fat-body particles. Particle suspensions in 0·25 M-sucrose, equivalent to about 12% wet wt. of fat body, were incubated with TPNH and mM-schradan, pH 7·4, at 37° for 30 min., and the residual esterase activity was then determined. The two curves were obtained with two different fat-body preparations, the lower one (●) having stood overnight at 2° before use.

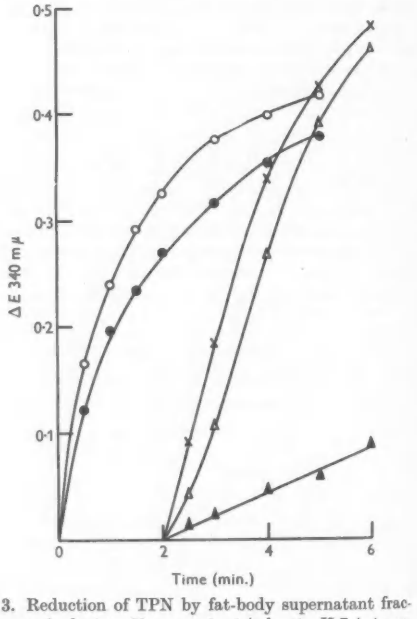


Fig. 3. Reduction of TPN by fat-body supernatant fraction equivalent to 20 mg. wet wt./ml., at pH 7·4, temp. 20°. TPN (mM) was added at zero time in the presence (○) and in the absence (●) of $MgSO_4$ (10 mM). The dialysed supernatant was inactive until glucose 6-phosphate (×), D-*iso*citrate (△) or L-malate (▲), all 5 mM, were added, at 2 min.

extent of about 2 mM in order to regain full activity, although the TPN-reducing power of the pig-heart *iso*citrate system was much greater than that of the supernatant. Therefore an attempt was made to estimate the amount of endogenous TPNH. Optical density at 340 mμ indicated a maximum possible concentration of 0·24 mM in the supernatant from a 200 mg./ml. homogenate. TPN was added to a supernatant preparation buffered with 1% $NaHCO_3$ saturated with CO_2, pH 7. When the optical density at 340 mμ had risen to a maximum, excess of lithium pyruvate was added, and the optical density declined as the reaction

$$malate^{2-} + TPN^{+} \rightarrow pyruvate^{-} + CO_2 + TPNH$$

was reversed. Similarly, when pyruvate was added to the supernatant without TPN, a small decline in optical density occurred (Fig. 4), and a calculation based on this change showed an approximate concentration of 45 μM-TPNH, or about 18 μM in a typical reaction mixture. Thus there seems to be a discrepancy factor of more than 10 between the endogenous TPNH and the amount that had to be added to replace it. Possibly the higher efficiency of the natural coenzyme can be explained in terms of binding and orientation.

Function of calcium and magnesium. $CaCl_2$ (mM) and $MgSO_4$ (10 mM) (see Fig. 3) both caused a small increase in the rate of reduction of TPN by the supernatant. In an experiment to test the effect of these metal ions on the particle fraction, the particle concentration was reduced, in the presence of a constant concentration of added TPNH, until it became rate-limiting. Under these conditions, neither $CaCl_2$ nor $MgSO_4$ had any effect on the rate of schradan conversion.

Inhibitors of conversion. The effects of inhibitors (brought to pH 7·4) were estimated by the percentage reduction of log (100/*b*) they caused. The results with whole suspensions are shown in Table 2, and with TPNH-particle preparations in Table 3. The inhibition by *p*-chloromercuribenzoate was reversed by mM-glutathione. Adenosine triphosphate (2 mM) did not restore the activity after treatment with 2:4-dinitrophenol.

Nature of the particle-bound enzyme. In view of the inhibitory effects of chelating agents and mepacrine (quinacrine, atabrine) a number of unsuccessful attempts were made to demonstrate

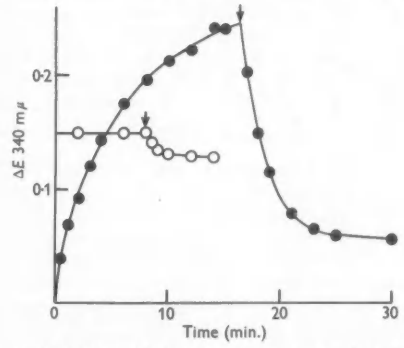


Fig. 4. Oxidation of TPNH in the supernatant fraction. ●, TPN (mM) was added at zero time to a fat-body supernatant buffered with $NaHCO_3$–CO_2, pH 7; ○, no TPN added. Arrows indicate the points at which pyruvate (10 mM) was added.

Table 2. *Inhibitors of schradan conversion in fat-body homogenates*

Inhibitors were pre-incubated for 5 min. at 37°, pH 7·4, with the fat-body preparation (centrifuged 2 min. at 600 *g* in 0·25 M-sucrose + 10 mM-$MgSO_4$), before addition of mM-schradan. The remaining esterase activity was determined after a further 30 min. at 37°.

Inhibitor	Concn. of inhibitor (mM)	Inhibition of schradan conversion* (%)
Anaerobiosis	—	100
8-Hydroxyquinoline	0·25	29
	1·0	83
αα′-Dipyridyl	0·5	44
	1·0	74
Diethyldithiocarbamate	0·25	39
	1·0	74
Ethylenediaminetetra-acetate (EDTA)	1·0	22
	5·0	64
Potassium cyanide	5·0	0
Pyrophosphate	1·0	0
Citrate	1·0	0
Mepacrine	0·05	31
	0·25	73
p-Chloromercuribenzoate	0·25	53
	0·5	100
2:4-Dinitrophenol	0·05	15
	0·5	50
Antimycin A	2 μg./ml.	0

* Estimated by percentage decrease of log (100/*b*).

Table 3. *Inhibitors of schradan conversion by the fat-body particulate fraction*

The particles were fortified with enzymically prepared TPNH (0·5 mM final concn.), and incubated with inhibitor and schradan (mM), pH 7·4, at 37° for 30 min. before measuring residual esterase activity.

Inhibitor	Concn. of inhibitor (mM)	Inhibition of schradan conversion* (%)
αα′-Dipyridyl	1	97
Mepacrine	0·25	75
p-Chloromercuribenzoate	0·25	100
2:4-Dinitrophenol	0·5	61

* Estimated by percentage decrease in log (100/*b*).

the participation of a heavy metal, and of a flavine prosthetic group. For example:

(i) A fat-body homogenate, buffered with tris buffer, pH 7·4, was treated with $\alpha\alpha'$-dipyridyl followed by $FeSO_4$ at twice the concentration. No restoration of activity occurred.

(ii) A particle suspension was treated with ethylenediaminetetra-acetate (EDTA) and then dialysed for 3 hr. at 2° against isotonic sucrose. All activity (in combination with supernatant fraction) was lost and none was restored by adding Fe^{2+}, Fe^{3+} or Cu^{2+} ions. It was subsequently found that mere dialysis of a particle suspension against sucrose soln. for 3 hr. inactivated it (the presence of pieces of dialysis tube in the control suspension had no effect). This was not due to removal of cofactors, since washing the particles once in sucrose soln. and re-sedimenting and suspending them did not reduce their activity. A brief 45 min. dialysis of particles against 25 mM-EDTA caused only a very small decline in activity. Therefore the technique of dialysis was abandoned.

(iii) A homogenate was treated with dipyridyl sufficient to inhibit almost completely its schradan conversion. The particles were then separated by centrifuging and resuspended in fresh sucrose soln., in the hope that the metal, together with the excess of dipyridyl, would have been removed. However, when the particles were tested, in combination with untreated supernatant, their activity was found to be normal. Evidently the dipyridyl is weakly bound to the enzyme molecule and its inhibition is readily reversible. 8-Hydroxyquinoline inhibition was also reversed by washing.

(iv) FAD (0·4 mM) had no effect on the activity of a fat-body homogenate. The effect of mepacrine (0·25 mM) was not reduced by the previous addition of FAD or FMN (0·25 mM).

(v) Homogenates were incubated at various acid pH values before restoring to 7·4 and measuring schradan conversion. Pre-incubation for 6 min. at 37° and pH 4·4 was needed to cause a substantial reduction of activity, but it was not restored by Fe^{2+} ions (0·5 mM) or FAD (0·1 mM) or both.

Non-enzymic activation

A reaction mixture consisting of ascorbic acid, EDTA and ferrous (or ferric) iron, which accomplishes certain aromatic hydroxylations, has been described by Udenfriend, Clarke, Axelrod & Brodie (1954). This system also produced an esterase inhibitor when incubated with schradan, and controls showed that ascorbic acid was not essential.

Convenient concentrations were: $FeSO_4$ (5 mM), EDTA (5 mM), schradan (20 mM). EDTA solution was neutralized with NaOH, and the system was buffered with phosphate buffer, pH 7, and shaken in air. The product of this reaction was applied at 1/20 final dilution to an esterase preparation to assess its inhibitory action. The amount of EDTA added was just sufficient to prevent precipitation of the iron by the phosphate buffer.

The following facts were observed:

(i) Fe^{2+} ions could not be replaced by Fe^{3+}, Cu^{2+}, Co^{2+} or MoO_4^{2-} ions, nor had ferrocyanide any action on schradan.

(ii) Production of inhibitor stopped within 3 min. at 37°, 8 min. at 22° (Fig. 5) or 16 min. at 2°.

(iii) Much less inhibitor was formed if the reaction occurred in an evacuated Thunberg tube.

(iv) If $FeSO_4$ was pre-incubated for 5 min. with EDTA in air before adding schradan, no inhibitor was formed, but if the pre-incubation was anaerobic, inhibitor formation was normal. When $FeSO_4$ was tipped into EDTA solution from the side arm of a Warburg flask, a rapid uptake of O_2 occurred, to the extent of 1 equivalent of O_2/atom of Fe (Fig. 5). If the molecular ratio of EDTA to Fe^{2+} ions was reduced to less than one, consumption of O_2 was limited to 1 equivalent/molecule of EDTA.

(v) In the presence of $FeSO_4$, unchelated and unbuffered, some inhibitor was produced from schradan, and pre-incubation of the $FeSO_4$ in air had no effect. However, the amount of inhibitor detectable again quickly reached a steady level, possibly due to the setting up of an equilibrium, the inhibitor being unstable at the low pH involved.

(vi) Reducing agents were added in the hope of promoting the reaction, but metabisulphite pre-

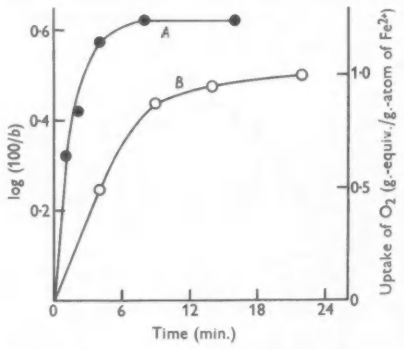


Fig. 5. Model system. Curve *A*: units of inhibitor produced (*b* = percentage of initial esterase remaining after incubating with inhibitor for 15 min. at 37°). EDTA (5 mM), schradan (20 mM), $FeSO_4$ (5 mM) were added at zero time. pH 7, temp. 22°. Curve *B*: rate of oxygen uptake. EDTA (10 mM), $FeSO_4$ (10 mM) were added at zero time, pH 7, temp. 20°.

vented, and thiosulphate reduced, the formation of inhibitor. Ascorbic acid (40 mM), at pH 5·7 and 37°, increased the inhibitor production, but did not prolong it beyond 3 min. When ascorbic acid was tipped, in a Warburg flask, into a Fe^{2+}ion–EDTA mixture in which uptake of O_2 had ceased, a further rapid uptake occurred, equivalent to complete oxidation of the ascorbic acid to dehydroascorbic acid.

A Co^{2+} ion–2 histidine chelate, which combines reversibly with molecular O_2 (see Martell & Calvin, 1952), was ineffective in producing an esterase inhibitor from schradan.

Dimefox

A few experiments were carried out with dimefox in order to compare its behaviour with that of schradan. It was also converted into an anti-esterase by the particles of the fat body fortified with TPNH, and the converting power of eighth-day fat body was considerably lower than that of fifth-day fifth-instar. However, a given concentration of dimefox caused the same degree of esterase inhibition in intact or homogenized fat body as did approx. 50 times the molar concentration of schradan, although in rat liver equal concentrations of the two compounds produce about the same effect (Fenwick *et al.* 1957).

Experiments in vivo

The LD_{50} values for schradan and dimefox administered by intrathoracic injection of solutions in 'insect Ringer' (0·01 ml./g. body wt.) were approx. 1800 and 45 μg./g. respectively, with young-adult locusts.

There was no appreciable difference in toxicity of dimefox between young adults and fifth-day fifth-instar nymphs. With schradan, the nymphs were probably slightly less susceptible than adults. An injection of 2000 μg./g. caused the death of 70% young adults and 30% of fifth-day fifth-instar nymphs.

DISCUSSION

It has been shown that in locust fat body, as in rat liver, the production of an esterase inhibitor from schradan and dimefox depends upon the presence of the particulate and soluble fractions of the cell contents, requires oxygen, and is activated by Ca^{2+} or Mg^{2+} ions. The soluble supernatant can be replaced by TPNH (as distinct from DPNH in the rat), and contains at least three enzymes capable of catalysing the reduction of TPN, namely glucose 6-phosphate and *iso*citric dehydrogenases and malic enzyme. The function of Ca^{2+} and Mg^{2+} ions is probably to activate these enzymes.

This strange need for a reducing agent in an oxidative process has been discussed in a review article by Brodie (1956), in connexion with the metabolism of various drugs. It was suggested that a peroxide-like compound, formed during the oxidation of TPNH, might then oxidize the drug. O'Brien (1957) demonstrated the formation of hydrogen peroxide on the addition of DPNH to mouse-liver homogenates, but could not correlate the rate of its formation with that of schradan conversion. Furthermore, hydrogen peroxide would not replace DPNH in fortifying a microsome suspension, although if catalase was added as well, conversion of schradan did occur.

Mason, Fowlks & Peterson (1955) proposed that the Cu^{+} ions of phenolase are oxidized to Cu^{2+} ions during the process of transfer of oxygen to the substrate. A reducing system would be needed to regenerate the active form of the enzyme, and Velick (1956) suggested that this might also be the function of TPNH in Brodie's (1956) drug-metabolizing systems, if the reduced form of a metallo-enzyme were involved.

It was therefore interesting to observe that the conversion of schradan by the intracellular particles of locust fat body, supplemented with TPNH, was prevented by $\alpha\alpha'$-dipyridyl. It was hoped that chelating agents would provide a means of removing the supposed metal from the enzyme, and that its activity would then be restored by adding the appropriate metal ion, as has been done with a number of metallo-enzymes. However, the inhibition proved to be reversible by washing the particles, so presumably the metal is not removed, but merely blocked, by the chelating agents used. A similar phenomenon occurred in the inhibition of copper-containing uricase by potassium cyanide, which was reversed by dialysis (Mahler, Hübscher & Baum, 1955). Nor was the commonly used technique of dialysis against cyanide solution of value, since (*a*) dialysis inactivated the particle suspension, conceivably owing to a lowering of pH at the dialysis membrane (Gordon, 1957), and (*b*) cyanide did not inhibit the conversion of schradan. The lack of inhibition by cyanide, pyrophosphate or citrate cannot be used as an indication of the nature of the enzyme system, since the permeability of the particle membrane to these materials is unknown.

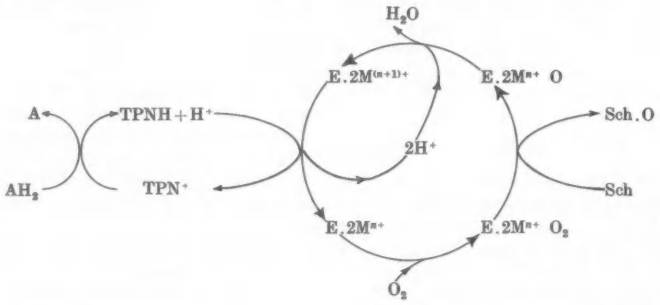
Although no direct evidence for the participation of a metal in the natural system could be obtained, it has been shown that Fe^{2+} ions, particularly in the form of a chelated complex with EDTA, in equimolecular proportions, can catalyse the oxidative formation of an esterase inhibitor from schradan. The reaction ceases as the complex is auto-oxidized (presumably to the ferric form), and Fe^{3+} ions among others are inactive, which is of particular interest in view of the need for a reducing agent in

animal systems. The addition of ascorbic acid enhanced inhibitor production, and it was itself oxidized, but ascorbic acid could not replace TPNH in the natural system. The spontaneous uptake of oxygen by a Fe^{2+} ion–EDTA complex may have some practical interest in dealing with an enzyme whose activity is measured manometrically (see, for example, Schepartz, 1953). If Fe^{2+} ion is added to an enzyme that has been inhibited by EDTA, a brief uptake of oxygen will occur which should not be confused with the restoration of enzyme activity.

Mepacrine inhibits enzymes with FMN and FAD prosthetic groups (Haas, 1944; Mackler, Mahler & Green, 1954), and was shown by Haas (1944) to compete with FMN for the apoenzyme of TPN cytochrome *c* reductase. The inhibition was irreversible, but could be prevented by the previous addition of small amounts of prosthetic group. The effect of mepacrine on schradan conversion was not changed by the presence of FAD or FMN, but again such negative results must be assessed with caution in view of the particle-bound nature of the system.

The fact that inhibition of conversion by *p*-chloromercuribenzoate can be reversed by glutathione (cf. Mahler, 1954) may indicate that the vital –SH groups are concerned with orientating the substrate on the enzyme surface rather than binding prosthetic groups.

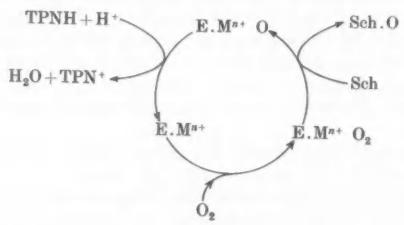
It seems probable that further information about the nature of this oxidizing system must await its solubilization and purification, but in the meantime it is possible to surmise that the mechanism is of the type discussed by Mason *et al.* (1955), with the possible participation of a flavin nucleotide as an intermediate hydrogen carrier:



representing the net reaction:

$$Sch + O_2 + AH_2 \rightarrow Sch.O + H_2O + A,$$

where AH_2 is an oxidizable substrate such as glucose 6-phosphate, E is the enzyme protein, M is a metal ion and Sch.O is the esterase inhibitor, probably the methylol derivative of schradan (Spencer & O'Brien, 1957). Or alternatively, without a change of valency of the metal ion:



The large difference in toxicity between schradan and dimefox may be connected with the much greater overall efficiency of dimefox conversion with esterase-inhibition in fat-body preparations *in vitro*, if this implies a similar high efficiency in other tissues, e.g. the nerve cord. It has not been determined whether the higher efficiency is due to faster conversion, slower destruction of the product as compared with rat liver, or a higher susceptibility of the esterase. It is not likely that conversion in the fat body contributes materially to the lethal effect *in vivo*, since considerable variations in fat-body activity are not related to changes in toxicity.

SUMMARY

1. The capacity of the fat body of the desert locust to convert schradan into an esterase inhibitor is high in young fifth-instar nymphs, but falls sharply before the final moult.

2. The conversion requires oxygen, reduced triphosphopyridine nucleotide and a particle-bound enzyme system.

3. It is inhibited by chelating agents (reversibly), and by mepacrine, *p*-chloromercuribenzoate and 2:4-dinitrophenol.

4. No requirement for a metal or flavine prosthetic group could be shown with the particle-bound system.

5. A Fe^{2+} ion–ethylenediaminetetra-acetic acid complex can catalyse the oxidative formation of an inhibitor from schradan.

6. A mechanism for the simultaneous oxidation of schradan and reduced triphosphopyridine nucleotide is suggested.

7. The requirements for the conversion of dimefox to an anti-esterase are the same as those for schradan, but the rate of esterase inhibition is much higher. Dimefox is also considerably more toxic than schradan to the locust, but LD_{50} does not vary with fat-body converting activity.

I wish to express my thanks to Dr B. A. Kilby for his help in frequent discussions. I am indebted also to the directors of Fisons Ltd. and to the Agricultural Research Council for financial support; and to the Anti-Locust Research Centre for a regular supply of locusts.

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Cytochromes and some Respiratory Enzymes in Mitochondria from the Spadix of *Arum maculatum*

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The discovery by James & Beevers (1950) that the rapid respiration of the *Arum* spadix is unaffected by cyanide stimulated interest in the mitochondria of this tissue. Mitochondria isolated from the spadix of *Arum maculatum* have been shown to be similar to those obtained from many other plant and animal tissues in that they are able to oxidize intermediates of the citric acid cycle (Hackett & Simon, 1954; James & Elliott, 1955) and contain cytochromes *a*, *b* and *c* (Bendall & Hill, 1956). However, these oxidations are inhibited to only a small extent by cyanide (James & Elliott, 1955) and are exceptionally rapid. It has also been shown (Bendall & Hill, 1956) that in addition to the normal cytochrome system *Arum* mitochondria contain a relatively large amount of cytochrome b_7, which can be oxidized by air in the presence of cyanide. It was suggested that cytochrome b_7 accounts for a significant part of the cyanide-stable respiration of the mitochondria.

Simon (1957) has recently measured the rates of oxidation of succinate and cytochrome *c* at different stages of development of the spadix, and determined the effect of cyanide and antimycin A on these enzyme systems. He has shown that the succinic-oxidation system rapidly increases in activity as the spadix grows and matures, and that in the mature spadix cytochrome *c* oxidase and the succinic system are about equally active, despite the comparatively small effect of cyanide on the succinic system.

Further studies on the respiratory enzymes and cytochromes in *Arum* mitochondria are described in the present paper. The effects of cyanide and

antimycin A were investigated in some detail, and the properties of mitochondria from young and mature spadices have been compared.

MATERIALS AND METHODS

Plant material. Inflorescences of *Arum maculatum* were collected from wild colonies near Cambridge either when still growing, when the spathe was approx. 8 cm. tall and only just emerging, or just before the opening of the spathe. They were either used immediately or stored at 4°. The experimental material was the sterile head of the spadix, after removal of the papillae and coloured cell contents of the epidermis in the mature spadices. Young spadices which had already become pigmented were rejected.

On the suggestion of Dr C. T. Prime corms of *A. neglectum* (Towns.) Ridley (*A. italicum* var. *neglectum* Towns.; *A. italicum* auct. angl. non Mill.) were collected in August 1954 from a colony at Arundel. The plants were grown in the University Botanic Gardens by courtesy of Mr J. S. L. Gilmour. The inflorescences were harvested just before the opening of the spathe and stored as above.

Peas (*Pisum sativum* var. Gradus) were purchased locally. They were washed under a running tap for half an hour, and soaked in water for 6 hr. and germinated in moist vermiculite for 3½ days in the dark at room temperature (about 25°).

Preparation of mitochondrial fractions. The method of preparation of *Arum* mitochondrial fractions was the same as that described in a previous paper (Bendall & Hill, 1956). The washed mitochondria were suspended in 0·20 M-sucrose containing 0·05 M-phosphate (KH_2PO_4–Na_2HPO_4), pH 7·1, for spectroscopic experiments. For manometric experiments they were suspended in 0·25 M-sucrose before addition to the manometer vessels.

In the preparation of mitochondrial fractions from pea seedlings the cotyledons only were used. Tissue (30 g.) was ground with 40 ml. of 0·4 M-sucrose containing 0·1 M-phosphate (KH_2PO_4–Na_2HPO_4), pH 7·1. The brei was strained through muslin and centrifuged for 5 min. at 1700 *g* in an International refrigerated centrifuge, model PR-1, which precipitated mainly starch grains. The supernatant was centrifuged for 15 min. at 14 000 *g* to give the mitochondrial fraction. The mitochondria were washed once by suspending the pellet in the grinding medium and recentrifuging. The washed mitochondria were then suspended in a small volume of grinding medium.

Rates of oxygen uptake by mitochondria. Uptake of O_2 was followed for 1 hr. in Warburg manometers at 20°. The rates were fairly steady, but usually fell off a little during the second half hour; initial rates only are quoted. Carbon dioxide was absorbed with KOH and filter paper in the centre well, or with the $Ca(CN)_2$–$Ca(OH)_2$ mixtures described by Robbie (1948) when cyanide was present in the reaction mixture.

The reactions were started by adding the mitochondrial suspension to the flasks before attaching them to the manometers. Cyanide and antimycin A were added after the mitochondria.

Convenient rates of uptake of O_2 were obtained for measurement of rates of succinate oxidation when mitochondria prepared from 0·1 g. of mature spadix or 1 g. of young spadix were used in each manometer flask. These weights are rough indications only, and the actual quantity of mitochondria used varied somewhat from one experiment to another. A quantitative recovery of mitochondria was not attempted. The endogenous uptake of O_2 of washed mitochondria was less than 30 µl. of O_2/mg. of N/hr. for mature spadices, and less than 10 µl. of O_2/mg. of N/hr. for young spadices.

Oxygen uptake of tissue slices. A large number of slices were cut about 0·2 mm. thick with the aid of a hand microtome, and suspended in 0·05 M-phosphate (KH_2PO_4–Na_2HPO_4), pH 7·1, and washed in the same buffer. Slices (50) were transferred to each manometer flask, which contained 3 ml. of 0·05 M-phosphate, pH 7·1, or 3 ml. of buffer containing mM-HCN in the main compartment, and 20% (w/v) KOH or the appropriate $Ca(CN_2)$–$Ca(OH)_2$ mixture in the centre well. The temperature was 30°.

Succinic-cytochrome c reductase activity. This was measured spectrophotometrically by following the reduction of cytochrome *c* at 550 mµ in a Beckman spectrophotometer model DU at room temperature (about 20°). A value of 18·5 was used for the difference between ϵ_{mM} of the reduced and oxidized forms (Margoliash, 1954). HCN (mM) was added to the reaction mixture to inhibit cytochrome *c* oxidase. In a few cases HCN was omitted and the reaction was carried out in an evacuated Thunberg tube specially made for use in a spectrophotometer (obtainable from the Thermal Syndicate Ltd., Wallsend, Northumberland). Small differences in rate were observed between the anaerobic and cyanide-treated particles, but the results were not consistent and HCN was normally added for convenience. The optical density of the experimental cell was measured against a blank containing mitochondria and all reagents except cytochrome *c*.

Spectroscopic observations. These were made with a Zeiss microspectroscope ocular. The scale was set with the α-band of reduced cytochrome *c* at 550 mµ. For observations at room temperature the mitochondrial suspensions, about 5–10 g. of fresh tissue/ml., were held in a 1 cm. glass cell with optical faces. For observation of spectra at the temperature of liquid N_2 the mitochondrial suspension was mixed with an equal volume of glycerol and examined as described by Keilin & Hartree (1949).

Cytochromes were usually reduced by the addition either of one drop of M-sodium succinate, pH 7·1, or of a few crystals of sodium dithionite to 1·5–2·0 ml. of mitochondrial suspension.

Cytochrome concentrations. The intensities of the α-bands of cytochrome were measured by comparison with suitable standards in the spectrocolorimeter of Hill (1936).

For cytochrome *c* the standard was a solution of purified heart-muscle cytochrome *c* which was estimated by determination of the extinction at 550 mµ with a Beckman spectrophotometer after addition of $Na_2S_2O_4$. A molar-extinction coefficient of $27{\cdot}7 \times 10^3$ l. moles^{-1} cm.^{2} was used (Margoliash, 1954).

Cytochrome *b* was compared with the α-band of cytochrome *b* in a suspension of baker's yeast in saturated sucrose solution in the colorimeter cup. It was judged that the intensity of the *b* band in the yeast suspension was 70% of that of the *c* band. It was then assumed that the millimolar extinction coefficients of yeast cytochrome *b* and *Arum* cytochromes *b* and b_7 are equal and have a value of 33, which is the value for pyridine haemochromogen

(Lemberg & Legge, 1949) and crystalline yeast cytochrome b_2 (Appelby & Morton, 1954).

At the suggestion of Dr R. Hill cytochromes $a+a_3$ were estimated by comparison with the band at 605 mμ of a dilute solution of a chlorinated resorufin derivative, which was probably the tetrachloro compound. The dye was prepared by Dr Hill by the following method. Resorufin (10 mg.) was suspended in 5 ml. of water and dissolved by the addition of 0·5 ml. of 0·1 N-NaOH. An aqueous solution (100 ml.) containing 1% (w/v) of $NaHCO_3$ and 1% (w/v) of KCl was added. The mixture was treated at room temperature during 20 min. with excess of sodium hypochlorite solution (0·4 ml., 10–14% of available chlorine). The solution should be between pH 8 and 8·55. The sparingly soluble potassium salt of chlororesorufin was centrifuged off and washed twice with the $NaHCO_3$–KCl mixture. It was reprecipitated by dissolving in 50 ml. of boiling water, followed by the addition of 0·5 g. of KCl and cooling. For use as a standard the potassium salt was dissolved in 90% acetone containing a trace of $NaHCO_3$, and about an equal volume of methanol was added to bring the position of the main absorption band to the desired value in the region of 605 mμ. The extinction at the peak of the band was determined spectrophotometrically, and the value of 20 given by Dannenberg & Kiese (1952) for the millimolar extinction coefficient of heart-muscle cytochrome *a* at 606 mμ was used.

The above method of estimating cytochromes $a+a_3$ was not available in earlier experiments. In these, yeast suspensions were used as standards and their cytochrome *c* contents were estimated by comparison with purified cytochrome *c*. It was assumed that the ratio of *c*:*a* is approximately constant in different samples of yeast and has the value 1·1, which is the mean of two values (0·99 and 1·21) obtained by comparison with cytochrome *c* and chlororesorufin.

The maxima of absorption bands were matched. The methods for the estimation of cytochromes *c* and $b+b_7$ do not allow for the contributions from minor components with absorption bands at 563 and 553 mμ at $-196°$ (see Results).

The absolute cytochrome concentrations expressed in terms of mitochondrial nitrogen are only approximate because multiple reflexions in the turbid suspensions may cause an error in the estimation of the light path (Holton, 1955).

Nitrogen. The nitrogen content of mitochondrial suspensions was determined by micro-Kjeldahl digestion as described by Chibnall, Rees & Williams (1943), followed by distillation and titration.

Dry weights. Dry weights of tissue slices and whole-spadix appendages were determined after drying in an oven at 105°.

Reagents. Barium adenosine triphosphate was prepared from muscle by the late Mr E. J. Morgan by a method based on that of Needham (1942). Solutions of sodium adenosine triphosphate (0·05 M) were prepared by the method of Bailey (1942) and stored at $-20°$. In some experiments disodium adenosine triphosphate supplied by Pabst Laboratories, Milwaukee, Wis., U.S.A., was used.

Cytochrome *c* was prepared by Mr D. C. Gardiner by the method of Keilin & Hartree (1945) up to the 0·34% Fe stage.

A concentrated solution of A.R. succinic acid was neutralized with NaOH, treated with 8-hydroxyquinoline to remove heavy metals (Chappell & Perry, 1953), and the disodium salt was precipitated with ethanol. α-Oxoglutaric acid, which was prepared by Mr B. R. Slater, contained approx. 0·5% of succinic acid, as measured with a Keilin & Hartree (1947) heart-muscle preparation. L-Malic acid was supplied by L. Light and Co. Ltd.

Crystalline antimycin A was supplied by the Wisconsin Alumni Research Foundation. Stock solutions (100 and 500 μg./ml.) in 96% ethanol were stored at 4°.

RESULTS

Cytochrome components

The cytochrome spectrum of mitochondria from the mature spadix has already been described (Bendall & Hill, 1956). In the presence of sodium dithionite the α-bands of cytochromes *a* and *c* can be seen, and also a strong band at 560 mμ which is due mainly to cytochrome b_7 (Fig. 1*a*). When a concentrated suspension of mitochondria from young spadices (10 g. of fresh tissue/ml.) was reduced with dithionite and examined with a microspectroscope the α-bands of cytochromes *a* and *c* were also visible, but the *b* band at 561 mμ was relatively weak compared with the corresponding band in the mature spadices, being now only slightly stronger than the *c* band (Fig. 1*c*).

This spectrum therefore more closely resembles the spectrum of mitochondria from germinating-pea cotyledons (Fig. 1*e*) than that of the mature *Arum* mitochondria. When mitochondria from the young spadix or from pea cotyledons were reduced with succinate instead of dithionite the *b* band was sharper and slightly less intense. The spectra were then similar to the spectrum of baker's yeast. A weak chlorophyll band was often visible at about 670 mμ in preparations from young *Arum* spadices.

In the presence of approx. mM-cyanide and sodium succinate (approx. 0·03 M) it was found possible to obtain partial oxidation of the *b* components of the young *Arum* mitochondria by shaking at 0° with air. This suggested that the young mitochondria also contain some cytochrome b_7.

The concentrations of cytochromes in young- and mature-spadix mitochondria are shown in Table 1. The scatter of results for the absolute concentrations in the young mitochondria do not allow any definite conclusions to be drawn concerning the changes of concentration as the spadix matures. However, the relative concentrations are more consistent and it is clear that the concentration of *b* components relative to that of cytochrome *c* increases about three times.

When a suspension of mature *Arum* mitochondria was reduced with sodium dithionite, and cooled in liquid nitrogen and rapidly examined with a micro-spectroscope, the absorption bands showed the

expected sharpening and were now located at 600, 558 and 549 mμ, with faint sharp bands also at 536 and 528 mμ (Fig. 1*b*). A faint satellite band of cytochrome *c* at about 546 mμ (Keilin & Hartree, 1949) could sometimes be seen, but it was not as distinct as in purified heart-muscle cytochrome *c* (see Estabrook, 1956). A faint sharp band could occasionally be seen at 563 mμ. When the *b* components were only partially reduced with succinate this band was more distinct, and at the same time another band appeared at 554 mμ, which may be due to cytochrome c_1.

The spectrum of young-spadix mitochondria reduced with sodium dithionite and cooled in liquid nitrogen was similar to the spectrum of the partially oxidized mature mitochondria (Fig. 1*d*). Pea-cotyledon mitochondria reduced with dithionite and examined in the same manner showed similar bands with slightly different relative intensities at 564, 558, 554 and 549–550 mμ (Fig. 1*f*).

The α-band of the reduced cytochrome a_3–cyanide complex was observed in *Arum* mitochondria in the following manner. A concentrated suspension of mature mitochondria was placed in a Thunberg tube, a little solid sodium succinate was added to the suspension, and a few milligrams of solid potassium cyanide were placed in the stopper. The tube was thoroughly evacuated and the cyanide tipped in. A shading extending to 590 mμ was now observed on the short-wavelength side of the α-band of cytochrome *a*. The shading was approximately half as intense as the band at 603 mμ, so the ratio a_3:*a* is probably smaller than in heart muscle. On admitting air to the tube the shading disappeared and did not reappear on addition of dithionite.

The spectrum of mitochondria from the spadix of *A. neglectum* was intermediate between the spectra of the mitochondria from young and mature spadices of *A. maculatum*. The *b* cytochrome components of mitochondria from *A. neglectum* could be completely oxidized in the presence of succinate and cyanide by shaking at 0° with air.

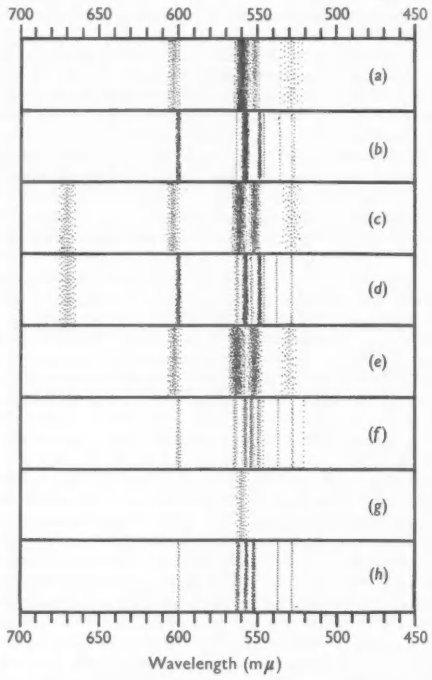

Fig. 1. Diagram showing the cytochrome spectra of *Arum* and pea-cotyledon mitochondria. (*a*) Mitochondria from mature spadices of *A. maculatum* + $Na_2S_2O_4$ at room temperature (20°). (*b*) The same at −196°. (*c*) Mitochondria from young spadices of *A. maculatum* + $Na_2S_2O_4$ at room temperature. (*d*) The same at −196°. (*e*) Mitochondria from cotyledons of germinating peas + $Na_2S_2O_4$ at room temperature. (*f*) The same at −196°. (*g*) Mitochondria from mature spadices of *A. maculatum* + succinate and antimycin A and shaken with air at room temperature. (*h*) The same after cooling to −196°. Effects of carotenoids and light scattering on the blue end of the spectrum are not shown. The band at 670 mμ in (*c*) and (*d*) is due to chlorophyll. Intensities of absorption bands may be compared within a particular spectrum, but not between spectra.

Table 1. *Cytochrome concentrations*

Results are expressed as μmoles of haem/g. of mitochondrial N. Cytochrome ($a+a_3$) concentrations shown in bold type were obtained indirectly by comparison with yeast (see Materials and Methods).

Date of experiment	Age of spadix	$a+a_3$	$b+b_7$	c	Ratio $(b+b_7)/c$
18 April 1955	Young	**3·6**	3·7	2·0	1·8
18 April 1956	Young	**0·97**	1·3	0·84	1·6
27 April 1956	Young	**1·6**	2·0	1·5	1·3
20 May 1955	Mature	**2·0**	3·6	0·74	4·9
2 June 1955*	Mature	**2·1**	4·2	0·79	5·3
7 June 1955*	Mature	**1·2**	2·1	0·64	3·3

* Inflorescences were collected 30 May 1955.

Table 2. *Effect of cyanide on the oxidation of citric acid-cycle intermediates*

Control rates of oxygen uptake are expressed as μl. of O_2/mg. of mitochondrial N/hr. (1 mg. of mitochondrial N corresponded to 2·56 g. of fresh young spadix and 1·30 g. of mature spadix). Each reaction vessel contained 0·2 M-sucrose, 0·05 M-phosphate (KH_2PO_4–Na_2HPO_4), pH 7·1, 0·33 mM-adenosine triphosphate, mM-$MgSO_4$, 0·03 M-substrate and mitochondria in a total volume of 3 ml. HCN, when present, was mM. Temperature, 20°.

	Young mitochondria		Mature mitochondria	
Substrate	Control	Inhibition (%)	Control	Inhibition (%)
Succinate	332	85	4650	27
L-Malate	77	−11	2090	64
α-Oxoglutarate	97	—	1540	—
Citrate	—	—	1080	36

Oxidation of intermediates of the citric acid cycle and inhibition by cyanide

The rapid rates at which *Arum* mitochondria oxidized intermediates of the citric acid cycle are shown in Table 2, together with the effect of cyanide on some of the systems. The activities of the mature mitochondria were exceptionally high. The mean value from thirteen experiments at 20° for the oxygen uptake with succinate as substrate was 4060 μl. of O_2/mg. of N/hr., which may be compared with 2190 μl. of O_2/mg. of N/hr. at 37° for liver mitochondria (Hogeboom, Schneider & Palade, 1948). The activities of mitochondria from the young spadices were always lower. Comparison of the rates of respiration of succinate by mitochondria from young spadices collected at different stages in the season suggested that there is a steady increase in activity as the spadix grows and matures. Only the extreme values are given in Table 2 since Simon (1957) has already published similar results. The same conclusion is reached whether the activities are expressed in terms of mitochondrial nitrogen (as in Table 2) or of weight of fresh spadix (Simon, 1957). At the same time as the rate of respiration of succinate increased, the inhibition by mM-cyanide decreased. Thus the activity of the cyanide-stable succinate-oxidation system increased even more rapidly than the total activity. Simon (1957) made a similar observation but the change in cyanide inhibition was smaller in his experiments (from 65% with young mitochondria to 55% with mature mitochondria). In the present work the average inhibition was 30% in 10 different preparations of mature mitochondria, which falls between the value of 6% obtained by James & Elliott (1955) and 55% by Simon (1957).

When malate was the substrate the cyanide inhibition behaved in the opposite manner, increasing as the spadix matured. However, again the cyanide-stable rate of oxidation increased, although not so rapidly as when succinate was the substrate.

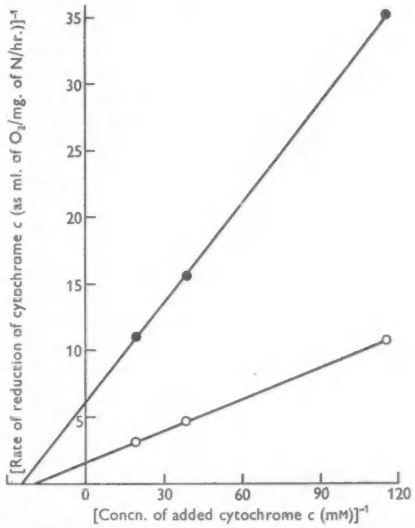

Fig. 2. Effect of cytochrome *c* concentration and pretreatment with water on the succinic-cytochrome *c* reductase activity of mature *Arum* mitochondria. ●, Mitochondria were suspended in 0·25 M-sucrose before the experiment, and 0·20 M-sucrose was present in the reaction mixture; ○, mitochondria suspended in 0·25 M-sucrose were diluted ten times with water and kept for 92 min. at 0° before starting the reaction, and sucrose was absent from the reaction mixture. Other components of the reaction mixture were 0·05 M-phosphate (KH_2PO_4–Na_2HPO_4), pH 7·1, 0·33 mM-adenosine triphosphate, mM-$MgSO_4$, 0·03 M-sodium succinate, mM-HCN, oxidized cytochrome *c* as indicated in the Figure and mitochondria in a total volume of 3 ml.

Effects of antimycin A

Succinic-cytochrome c reductase. The activity of this enzyme has been measured only in mitochondria from the mature spadix. The rates were

low unless the mitochondria were first treated with hypotonic solutions. When a mitochondrial suspension in 0·25 M-sucrose was diluted ten times with water, and kept at 0° for half an hour, the reaction velocity was increased several-fold. Pretreatment in water for longer periods produced only small further increases. When the reaction was started by addition of 0·1 ml. of mitochondrial suspension to the Beckman cell, sigmoid progress curves were sometimes obtained. The reason for the lag phase has not been ascertained, but it is unlikely to be due to reoxidation of cytochrome *c* by cytochrome *c* oxidase because mM-cyanide was present in the reaction mixture. The rate was linear when the reaction was started by the addition of succinate to the mitochondria which had been incubated with the reaction mixture for 5 min. at room temperature. The rate was dependent on the concentration of cytochrome *c*. The effects of pretreatment with water and cytochrome *c* concentration are shown in Fig. 2. In this experiment the reaction was started by addition of mitochondria, and the linear portions of the progress curves were used. The succinic-cytochrome *c* reductase activity at infinite cytochrome *c* concentration after pretreatment with water was 637 μl. of O_2/mg. of N/hr. The uptake of oxygen of the same mitochondrial preparation (without water pretreatment) in the presence of succinate was 3900 μl. of O_2/mg. of N/hr. The succinic-cytochrome *c* reductase could therefore account for only 16% of the uptake of oxygen, but it is not certain that the hypotonic treatment used completely activated the reductase system.

Antimycin A was found to cause 98% inhibition of the succinic-cytochrome *c* reductase at a concentration of approx. 0·3 μg. of antimycin/mg. of mitochondrial nitrogen (Table 3).

Respiration of succinate and malate. The effects of various concentrations of antimycin on the oxidation of these substrates are shown in Table 4. The

Table 3. *Effect of antimycin A on succinic-cytochrome c reductase of mature mitochondria*

Each reaction cell contained 0·2 M-sucrose, 0·05 M-phosphate (KH_2PO_4–Na_2HPO_4), pH 7·1, 0·33 mM-adenosine triphosphate, mM-$MgSO_4$, 0·03 M-sodium succinate, 52·1 μM-oxidized cytochrome *c*, mM-HCN and mitochondria in a total volume of 3 ml. Antimycin A was added in the form of a solution in 96% ethanol. The final concentration of ethanol was made 0·96% (v/v) in all cells. 1 mg. of mitochondrial N corresponded to 1·49 g. of fresh spadix. The rate in the absence of succinate was 0·8% of the rate in its presence. This endogenous rate was inhibited 50% by antimycin (0·0493 and 0·493 μg./mg. of N), and was not subtracted in calculating the percentage inhibition given below.

Concn. of antimycin A (μg./mg. of N)	Inhibition (%)
0·0493	0
0·148	65
0·493	98
1·48	98
14·8	98

Table 4. *Effect of antimycin A and cyanide on respiration of succinate and malate*

Reaction mixtures were the same as in Table 2. Antimycin A was added in the form of a solution in 96% ethanol. The final concentration of ethanol was made 1% (v/v) in Expt. 1, and 0·77% in Expts. 2 and 3. The ethanol alone caused 20% inhibition in Expt. 1, 9% in Expt. 2 and 11% in Expt. 3. These are not included in the percentage inhibitions given below. 1 mg. of mitochondrial N corresponded to 3·21 g. of fresh spadix in Expt. 1, 1·31 g. in Expt. 2 and 1·22 g. in Expt. 3.

Expt. no.	Age of spadix	Substrate	Concn. of antimycin A (μg./mg. of N)	HCN (mM)	Inhibition (%)
1	Young	Succinate	0·089	–	2
			0·445	–	39
			0·84	–	38
			8·9	–	47
			0	+	51
			0·89	+	56
2	Mature	Succinate	0·655	–	1
			1·31	–	7
			13·1	–	10
			218	–	40
			0	+	33
			13·1	+	40
3	Mature	L-Malate	0·122	–	1
			0·612	–	5
			6·12	–	10
			61·2	–	28
			0	+	56
			6·12	+	64

inhibitions were larger for the young mitochondria than the mature, but were always far from complete. The most significant concentration is about 0·3 μg./mg. of N, which gave maximal inhibition of the succinic-cytochrome *c* reductase. At this concentration there was no appreciable inhibition of the uptake of oxygen of the mature mitochondria with either succinate or malate as substrate.

Simon (1957) has also examined the effect of antimycin on the succinic oxidase system of mature *Arum*-spadix mitochondria. In contrast with the results described above he found that both mM-cyanide and antimycin in a concentration of about 3 μg./g. of fresh tissue gave about 50% inhibition, the antimycin inhibition being sometimes slightly higher than that of cyanide.

Spectroscopic experiments. When succinate is added to a concentrated suspension of mature *Arum*-spadix mitochondria the cytochromes become rapidly reduced and can be reoxidized by cooling to 0° and vigorously shaking with air (Bendall & Hill, 1956). At room temperature only a partial reoxidation of the *b* components can be obtained. However, when antimycin A (1 μg./g. of spadix) was added to the suspension cytochromes *a* and *c* could be oxidized at room temperature. Most of the *b* components were also oxidized but a faint broad band remained at 560 mμ (Fig. 1*g*). On standing, the cytochromes fairly rapidly became reduced again. It is concluded from this experiment that antimycin inhibited the reduction of cytochromes *a* and *c*, as in heart-muscle preparations (Chance, 1952) and liver mitochondria (Chance & Williams, 1955), but did not prevent the oxidation of a large proportion of the *b* components, as it does in the animal systems. The reduction of cytochromes *a* and *c*, which still occurred after the addition of antimycin, was probably due to traces of endogenous substrate and occurred after all the dissolved oxygen had been used up by the active antimycin-insensitive succinate-oxidation system.

The above experiment was repeated with a mitochondrial suspension in 50% (v/v) glycerol. In the presence of succinate, antimycin and air a faint broad band was again visible at 560 mμ. The suspension was then cooled in liquid nitrogen and the band was seen to split into three components of equal intensity at 563, 558 and 553 mμ (Fig. 1*h*). The suspension was then thawed and sodium dithionite added. Immediately cytochromes *a* and *c* became reduced and the *b* band intensified to its normal value.

The effect of antimycin A on the cytochrome system of the young mitochondria was qualitatively similar to its effect on the mature particles. This was also true of mitochondria from the spadix of *A. neglectum*.

When a concentrated suspension of washed mature mitochondria was allowed to stand at room temperature without any added substrate the cytochromes became reduced within a few minutes owing to endogenous substrate. With this low concentration of substrate it was possible to obtain complete oxidation of the *b* components of cytochrome in the presence of mM-cyanide, and the *a* and *c* bands were seen alone at 603 and 551 mμ. When this suspension was cooled in liquid nitrogen the only α-bands visible were those of cytochrome *a* at 600 mμ, and cytochrome *c* at 549 mμ, with a faint satellite band on the short-wavelength side. This indicated that it is possible to oxidize the components with bands at 563, 558 and 553 mμ in the presence of cyanide.

Since it is known that cytochrome b_7 can be oxidized directly by oxygen or by a pathway not involving cytochrome *c* oxidase (Bendall & Hill, 1956), it is probable that the *b* component which remains oxidized in the presence of antimycin A is b_7. The three bands which are visible in the presence of antimycin at the temperature of liquid nitrogen cannot be definitely identified yet, and their nature will be discussed later. Since antimycin inhibits the oxidation of cytochrome *b* in animal systems (Chance, 1952; Chance & Williams, 1955), it is probable that one of the components in *Arum* which remains reduced in the presence of antimycin is cytochrome *b*. This suggested that comparison of the intensity of the band at 560 mμ in the presence of succinate, antimycin and air, with its intensity in the presence of dithionite, would give a measure of the amount of cytochrome b_7 relative to that of the other components absorbing at 560 mμ, including cytochrome *b*. The results of such an experiment with both young and mature mitochondria are shown in Table 5. The proportion of the total absorption at 560 mμ due to cytochrome b_7 was 38% in the young mitochondria and 60% in the mature. It is noteworthy that in the same preparations the difference in the rates of respiration of succinate in the presence of antimycin was many times larger than the difference in b_7 content.

Respiration of the whole tissue

James & Beevers (1950) found that mM-cyanide had no appreciable effect on the respiration of slices of *Arum* spadix. With the youngest spadices examined they obtained only an 11% inhibition. This result was surprising, since the rate of respiration of succinate by mitochondria from young spadices can be inhibited as much as 80% and because young growing plant tissues are usually more sensitive to cyanide than old tissues. It was considered necessary to check their result because their method of determining the effect of cyanide was not entirely satisfactory. To avoid the

Table 5. *Concentrations of cytochrome* b_7 *and total* b *components in young and mature mitochondria*

Concentration of cytochrome b_7 was obtained by subtracting the intensity of the band at 560 mμ in the presence of excess of succinate, antimycin and air, from its intensity in the presence of $Na_2S_2O_4$. The antimycin concentration was 2·2 μg./mg. of N for the young mitochondria, and 3·9 μg./mg. of N for the mature mitochondria. The conditions for the measurement of rates of uptake of oxygen were as in Table 2.

Age of spadix	Concn. of cytochrome b_7 (μmoles of haem/g. of N)	Total concn. of *b* components (μmoles of haem/g. of N)	Rate of oxidation of succinate (μl. of O_2/mg. of N/hr.)
Young	0·93	2·45	280
Mature	1·53	2·55	3470

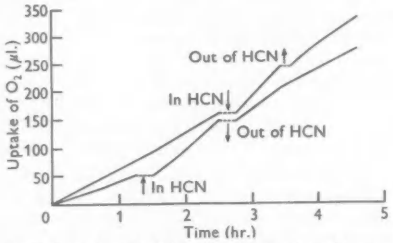

Fig. 3. Effect of mM-HCN on the respiration of two young spadices of *Arum maculatum*. Readings were taken every 5 min. Temperature, 20°.

difficulty of controlling the cyanide concentration in the manometer vessels while simultaneously absorbing the respiratory carbon dioxide, they soaked the slices in a cyanide solution for 2 hr. and then transferred them to manometer vessels containing potassium hydroxide in the centre well and no cyanide. The validity of this method depends on a slow rate of diffusion of hydrogen cyanide out of the slices and into the alkali in the centre well of the manometer flask. The rate of diffusion was not determined, but the respiration of a yeast suspension treated in the same manner was still completely inhibited 1 hr. after it was put in the manometer flasks. The experiment was therefore repeated with mM-cyanide in the main compartment of the manometer vessel and with a calcium cyanide–calcium hydroxide mixture (Robbie, 1948) in the centre well to absorb carbon dioxide and control the cyanide concentration. The cyanide stimulated the respiration by about 50%.

A similar result was obtained with the whole sterile appendages of young spadices, each appendage being placed in the centre well of a manometer flask. In this case the respiration of a spadix in the absence of cyanide was measured first, and then the spadix was transferred to a second manometer flask containing 0·1 ml. of mM-hydrogen cyanide in the centre well and 3 ml. of the appropriate calcium cyanide–calcium hydroxide mixture in the main compartment. The rate of oxygen uptake immediately increased to more than double the original value and continued steadily for 40–60 min. (Fig. 3). The spadix was then returned to the manometer vessel containing no cyanide but 20% potassium hydroxide in the main compartment. The rate of respiration slowly decreased but did not reach the original value during the course of the experiment. The Q_{O_2} (μl. of O_2/mg. dry wt./hr.) in the absence of cyanide was 2·1 at 20°.

The effect of cyanide on the mature spadix has not been redetermined.

DISCUSSION

Cytochrome components

No figures for cytochrome concentrations in mitochondria from sources other than *Arum* are apparently available in the literature, except for the relative concentrations given by Chance & Williams (1955). In the materials examined by these workers the cytochrome *b* concentration has always been found to be rather less than that of *c*. It may be calculated from the data given by Slater (1949) that a Keilin & Hartree (1947) heart-muscle preparation contains one to two times more *b* cytochrome, and approximately ten times more cytochrome *c*, than mature *Arum* mitochondria. On the other hand, the activity of the succinic oxidase system of *Arum* mitochondria is about four times greater than that of the heart-muscle preparation.

Arum mitochondria have been shown to contain cytochromes *a*, a_3, b_7 and *c*. In addition, a triple-banded spectrum, which probably cannot be ascribed to any of the above components, is visible in the presence of succinate, antimycin A and air. Of these three bands, the one at 553 mμ may be due to cytochrome c_1. The properties of cytochrome c_1 in heart-muscle preparations have been fully described and discussed by Keilin & Hartree (1955). It has been shown to be widespread in the animal kingdom and also occurs in yeast, but has not yet been described in higher plants apart from some indirect evidence obtained by Martin & Morton (1957). The evidence which suggests that the band

observed in *Arum* mitochondria at −196° at 553 mμ is due to cytochrome c_1 is as follows:

(i) The position is close to that described by Keilin & Hartree (1955), who give 553–554 mμ at room temperature and 551–552 mμ at liquid-air temperature. The difference may not be serious since it could be due to error in the estimation of the position. Thus the band appeared at 553 mμ in a heart-muscle preparation at −196° according to my estimation. It is also possible that small differences in technique, particularly in the nature of the suspending medium which was used, could cause variations in the band displacement at liquid-air temperature.

(ii) The *Arum* component was found to be thermolabile, as is heart-muscle c_1.

If cytochrome c_1 plays an essential role in the electron-transport system of mitochondria then it may be expected to occur in plants, since the fundamental similarity in function between animal and plant mitochondria has been established.

The evidence against identification of this component as cytochrome c_1 is that it is apparently auto-oxidizable (i.e. its oxidation can be observed in the presence of cyanide) and its reduction is not inhibited by antimycin. The former observation could be explained by supposing that cytochrome c_1 can be oxidized through cytochrome b_7 or some other cyanide-insensitive oxidase as well as through cytochrome *c* oxidase. The latter objection is more serious since it suggests a fundamental difference in the electron-transport system in the neighbourhood of the antimycin-inhibited factor between *Arum*-mitochondria and heart-muscle preparations. A definite conclusion cannot be reached until antimycin inhibition is better understood.

It is likely that one of the remaining two bands observed at −196° in the presence of antimycin is due to ordinary cytochrome *b*, because in animal systems the oxidation of cytochrome *b* is inhibited by antimycin (Chance, 1952; Chance & Williams, 1955). The third component remains unidentified. Since it has not been observed in animals it is possible that it does not play an essential part in the electron-transport system of the mitochondria, but is rather a haemochromogen precursor or degradation product of the cytochromes which for some reason tends to accumulate more in plants than in animals.

The differences in the cytochrome components of mitochondria from the young and mature spadices lie in the relative concentrations of *b* components. Whereas the mature mitochondria contain a relatively large amount of cytochrome b_7, the spectrum of the young mitochondria more closely resembles those of yeast and other plant mitochondria. The succinate-oxidation system insensitive to cyanide and antimycin increases more than ten times as the spadix matures, whereas the b_7 content only doubles. The significance of these differences is obscure because cytochrome b_7 is only one component in an electron-transport chain, and the activities of the other components have not been determined.

Respiratory chain in Arum *mitochondria*

Arum mitochondria possess the normal system for electron and hydrogen transport from substrates to oxygen via cytochromes *b*, *c*, *a* and a_3 and possibly c_1. In addition they possess at least one active alternative pathway which is not appreciably affected by antimycin, and at most only partially inhibited by mM-cyanide.

The succinic-cytochrome *c* reductase of *Arum* mitochondria was found to be almost completely inhibited by antimycin, and Simon (1957) has shown that mM-cyanide gives essentially complete inhibition of *Arum* cytochrome *c* oxidase. The uptake of oxygen by mature mitochondria with succinate as substrate was not significantly inhibited by a concentration of antimycin which completely inhibited the succinic-cytochrome *c* reductase. It is therefore probable that the oxidation system insensitive to cyanide and antimycin is capable of mediating almost all the uptake of oxygen for the oxidation of succinate to fumarate with mature mitochondria. Cyanide inhibited the respiration of succinate to a significantly greater extent than did the critical concentration of antimycin A. It is likely that the explanation of this lies partly in that a proportion of the observed uptake of oxygen in the presence of succinate is due to oxidation of malate formed from the succinate, and that oxidation of malate is inhibited to a large extent by cyanide.

The oxidation of a variety of substrates by intact liver mitochondria is completely inhibited by antimycin (Potter & Reif, 1952; Copenhaver & Lardy, 1952). This may be taken to mean that the cytochrome *c*–cytochrome oxidase pathway is completely inhibited; if this is also true of *Arum* mitochondria, the lack of inhibition of the respiration of malate by antimycin with mature mitochondria indicates that an alternative oxidation pathway plays a significant role in oxidation of malate. However, this is uncertain because of the large difference between the effects of cyanide and antimycin on oxidation of malate.

The larger effects of cyanide and antimycin on oxidation of succinate by young mitochondria indicate that as the spadix grows the alternative oxidase system increases relative to cytochrome *c* oxidase. This is supported by the demonstration by Simon (1957) that the cytochrome *c* oxidase activity decreases relative to the rate of respiration of succinate during this time.

Hackett (1957) has recently shown the presence of a pathway insensitive to cyanide and antimycin in mitochondria from the flowers of the skunk cabbage (*Symplocarpus foetidus*). Whether this system is significant in mitochondria from tissues other than aroid spadices is not yet known.

Function of the mitochondria in the respiration of the tissue

It is possible to estimate the importance of the mitochondria in the respiration of the whole tissue by a comparison of the rate of respiration of succinate by the mitochondria with the uptake of oxygen of the spadix. Even though no attempt at a quantitative recovery of mitochondria was made, the rate of oxidation of succinate by the mature mitochondria corresponded to a Q_{O_2} of 17 at 20°. According to James & Beevers (1950) the Q_{O_2} of the spadix may reach a value of 30 at 30°. It seems likely therefore that the mitochondria *in vivo* could account for all the rapid uptake of oxygen of the tissue. Furthermore, it was found that after centrifuging a concentrated homogenate of the mature spadix for 1 hr. at 100 000 *g*, only 1–2 % of the uptake of oxygen of the homogenate remained in the supernatant.

The Q_{O_2} of young mitochondria with succinate as substrate was variable, but was of the same order as the Q_{O_2} of the whole tissue. It is therefore surprising that whereas oxidation of succinate might be inhibited 80 % by cyanide, the respiration of the whole tissue was not inhibited at all, but stimulated. This apparent contradiction is unexplained.

SUMMARY

1. The cytochromes of mitochondria from young and mature spadices of *Arum maculatum* were compared. It was found that cytochrome b_7 occurs in young mitochondria, but in a smaller concentration relative to that of cytochrome *c* than in the mature.

2. A method is described for measuring the concentration of cytochromes $a+a_3$ in tissue preparations, by comparison with a chlorinated resorufin derivative. The concentrations of *a*, *b* and *c* cytochromes in *Arum* mitochondria were estimated.

3. The uptake of oxygen by *Arum*-spadix mitochondria in the presence of succinate or malate increased as the spadix matured.

4. Cyanide and antimycin A only partially inhibited the respiration of succinate and malate. Antimycin A gave smaller inhibitions than cyanide. It is suggested that there is an increase in the importance of the pathway insensitive to cyanide and antimycin relative to the cytochrome *c* oxidase pathway as the spadix matures.

5. The succinic-cytochrome *c* reductase of mature mitochondria was completely inhibited by antimycin A.

6. Antimycin A inhibited the oxidation of cytochromes *b* and two components with bands at 553 and 563 mμ. The oxidation of cytochrome b_7 was not inhibited.

7. The respiration of the whole young spadix was stimulated by mM-cyanide, even though the oxidation of succinate by the mitochondria could be inhibited as much as 80 %.

I wish to thank Dr R. Hill, F.R.S., for his continual interest, help and advice. I am also grateful to several other research workers, and especially to Dr H. E. Davenport and Professor D. Keilin, F.R.S., for helpful discussions. I am indebted to the Agricultural Research Council for a research studentship.

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The Chemical Composition and Structure of the Cell Wall of *Chlorella pyrenoidosa*

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The distribution of chemical substances in a complex organized structure such as a plant-cell wall can be studied by correlating a chemical analysis with a microscopical examination of the material. With green plants it is necessary to obtain a homogeneous tissue and to separate the cells before isolating the walls, and these are difficult experimental procedures. In the present work they have been avoided by using a unicellular green alga, *Chlorella*. It has been possible to isolate from this plant a cell-wall preparation which appears to be free from cell contents, and by using the electron microscope much of the fine detail of its structure has been shown. However, no histochemical stains, even of the limited specificity of those used in optical microscopy, are available for use with the electron microscope and it was difficult to assess the chemical nature of the structures observed. We have attempted to isolate the structural components from the wall and analyse them directly. To do this the wall has been degraded by chemical and enzymic methods which have destroyed one or more components but have left a discrete part of the wall which could be seen with the aid of the electron microscope.

The wall is composed of two distinct phases: an organized microfibrillar structure embedded in a continuous matrix, and it has been possible to separate these more or less intact one from the other. Treatment of the wall with dilute sodium or potassium hydroxide solutions removed the matrix and bundles of microfibrils were isolated. Incubation with an enzyme preparation made from the intestinal tract of the snail removed the microfibrillar elements and left a large part of the matrix material still in the shape of the original cell wall. In this way not only the chemical composition of the various parts of the wall were determined but also a more detailed knowledge of the distribution of the components was obtained.

The chemical composition of the cell walls of algae by direct isolation and chemical analysis has not previously been studied, although some indirect staining reactions and X-ray studies have been correlated with the cell-wall structure in certain species (Nicolai & Preston, 1952–53; Kreger, 1957).

EXPERIMENTAL AND RESULTS

Material used and general analytical methods

Alga. Chlorella pyrenoidosa was grown under sterile conditions on a completely defined salt medium (Emerson & Lewis, 1939) in large flasks (1–3 l.). A current of air + CO_2 (95:5) was passed through the medium (600 ml./min.) and growth was maintained for 10–14 days in daylight in a window facing west. The cells were harvested by centrifuging (1500 *g* for 20 min.), and washed with water and recentrifuged. Yield was approx. 0·7 g. dry wt./l.

Isolation of the cell-wall material by mechanical breakage of the cell. Chlorella cells (50 mg. dry wt.) were suspended in 10 ml. of water with 4 g. of fine glass beads (Ballotini no. 12, 0·15 mm. diam., Chance Bros. Ltd., Birmingham) and the mixture was placed in a vertical cup (internal measurements 5 cm. × 2·2 cm.) of a Mickle cell disintegrator (Mickle, 1948). Vibrations were continued for 90 min., after which the resultant suspension was treated as shown in Fig. 1, where the yields of the various fractions are also given. The temperature of the mixture during the breakage rose 15° from the initial room temperature.

Washed whole *Chlorella* cells centrifuged at 800 *g* for 10 min. and at 190 *g* for 30 min. came down completely into the sediment, leaving a clear colourless supernatant.

All the analyses were carried out on material dried at 0·01 mm. Hg over P_2O_5 at room temperature. Total N was determined by micro-Kjeldahl digestion (Chibnall, Rees & Williams, 1943) followed by distillation and titration. Total P was determined according to Fiske & Subbarow (1925).

Chromatography and electrophoresis of sugars. Descending chromatograms were run on Whatman no. 1 papers with pyridine–ethyl acetate–water (1:2:2, by vol.) for 14 hr. (Jermyn & Isherwood, 1949). Electrophoreses were run at 210 v in an apparatus similar to that described by Consden & Stanier (1952), with borate buffer, pH 9·2. The sugar spots were coloured in both procedures with aniline hydrogen phthalate (Partridge, 1949).

Chromatography of amino acids. Two-dimensional chromatograms were run in butanol–acetic acid–water (4:1:5, by vol.) and phenol buffered at pH 9·3 with borate according to Levy & Chung (1953). The spots were coloured with 0·3% triketohydrindene hydrate in butanol.

Chromatography of amino sugars. These were investigated by the methods described above for both sugars and amino acids. The spots were coloured by the spray reagents used for sugars and amino acids and also by the Elson–Morgan reagent described by Partridge (1948).

Enzyme preparation from Helix pomatia. The snails were dissected according to Keilin (1956) and the digestive tracts removed and cooled in ice. They were mixed with an equal volume of ice-cold water and crushed in a homogenizer with a Perspex pestle (Potter & Elvehjem, 1936). The resultant extract was dialysed against distilled water at 4° for 24 hr. and any precipitate which formed was removed by centrifuging at 15 000 *g* at 4° for 20 min.

This preparation has been quantitatively analysed and shown to contain only weak proteolytic activity but active lipases and carbohydrases. Some twenty different carbohydrases were examined, including cellulase, xylanase and mannanase (Myers & Northcote, 1958).

Chemical investigation of the cell wall

Elementary analysis and mineral content. The total N was 4·6%, and the total P 0·67% of the dry weight. When maintained at red heat (approx. 800°) in a platinum boat in a stream of clean dry air for 1 hr., 2·11 mg. of walls yielded a light yellow ash; 0·11 mg., 5·2%. This ash was dissolved in dil. HNO_3 and investigated chromatographically on Whatman no. 4 paper run with butanol saturated with N-HCl at 35° for 48 hr. The spots were detected by spraying with kojic acid and 8-hydroxyquinoline (Lederer & Lederer, 1955) and examination of the paper, before and after exposure to NH_3 vapour, under ultraviolet light. Iron, calcium and possibly aluminium were detected; no other spots were visible.

Lipid analysis. The lipid was determined by boiling the cell walls (55·1 mg.) with aq. 95% (v/v) methanol for 2·5 hr., and centrifuging and siphoning off the alcoholic supernatant from the walls, which were then redried at 0·01 mm. Hg over P_2O_5. The material was re-extracted with boiling dry ether for 2·5 hr. Yield of extracted cell walls was 50·1 mg. The alcohol and ether solutions were evaporated and the fat was weighed directly. Yield was 4·9 mg. (8·9%) and the wt. loss of the walls was 5·0 mg. (9·2%).

Acid hydrolysis. (i) For monosaccharide constituents. The whole cell walls (4 mg.) were hydrolysed by 2 ml. of 2N-H_2SO_4 for 6 hr. at 100°, then neutralized with $BaCO_3$ and filtered and the filtrate evaporated to dryness. Glucose, galactose, mannose, arabinose, xylose and rhamnose were identified in the hydrolysate by chromatographic and electrophoretic investigations.

(ii) For amino acids. Whole cell walls (4 mg.) were hydrolysed by boiling in 5 ml. of a mixture (1:1, v/v) of conc. HCl and formic acid for 24 hr. The hydrolysate was evaporated at 25° under vacuum, and dissolved in water and re-evaporated several times. Serine, glycine, glutamic acid, threonine, arginine, lysine, histidine, alanine, proline, tyrosine, valine, methionine, phenylalanine and leucine were identified in the hydrolysate.

(iii) For amino sugars. Whole cell walls (4 mg.) were hydrolysed by 1 ml. of 3N-HCl at 100° for 6 hr. The hydrolysate was evaporated at 25° under vacuum, dissolved in water and re-evaporated several times. The hydrolysate was finally dissolved in 5 ml. of 0·5N-HCl and put through an ion-exchange column (Dowex 50), which was washed with 10 ml. of water, and then the amino sugars were eluted with 2N-HCl and estimated according to Boas (1953). Calculated as glucosamine the yield was 3·3%. A neutral solution of the amino sugar was investigated chromatographically and electrophoretically and was identified as glucosamine.

Polysaccharides of the cell wall. Walls (50·1 mg.) from which fat had been extracted were treated with 10 ml. of

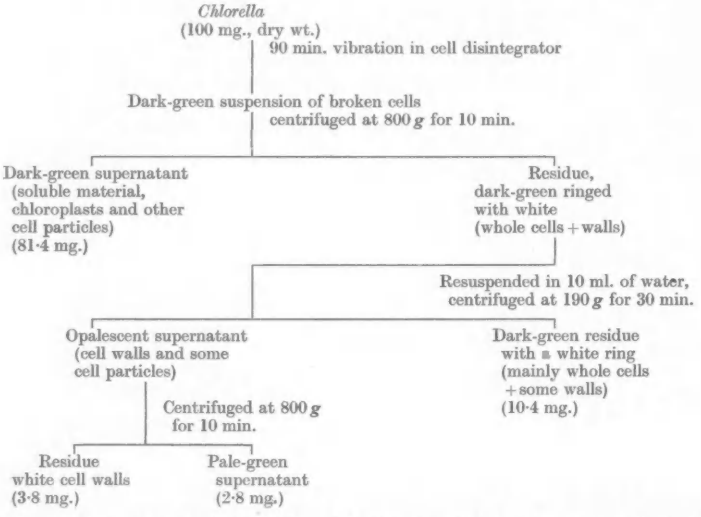

Fig. 1. Differential centrifuging of mechanically disintegrated *Chlorella* cells. The total recovery of material was 98·4 mg.

24% (w/v) KOH at room temperature under N_2 for 2 hr. The solution was siphoned off after centrifuging and the residue re-extracted with a further 10 ml. of KOH for 2 hr. The residue from this extraction was thoroughly washed with water by suspension and centrifuging. Normally six to eight washings were required to give a supernatant with the same pH value as the original wash water. The washings and alkali extracts were combined and the residual α-cellulose was dried over P_2O_5 at 0·01 mm. Hg. Yield of white preparation was 8·5 mg., 15·4% (N, 0·0%; ash, 0·05%).

The alkaline solution of hemicellulose was neutralized at 4° with cooled acetic acid and then evaporated under reduced pressure to half its volume. It was poured into a volume of ethanol so that the final concentration of alcohol was 85%, and the precipitate which formed was allowed to settle for 24 hr. This precipitate was centrifuged, washed with ethanol and dried. Yield, 34·1 mg. From a previous experiment it was known to contain mineral matter (approx. 27%) and N (approx. 3%) and a hydrolysate contained both amino acids and the amino sugar. It was therefore redissolved in water and reprecipitated with a mixture (1:4, v/v) of acetic acid and 85% ethanol. This precipitate was well washed with ethanol and dried. Yield of white powder was 17·1 mg., 31·0% (N, 0·2%; ash, 0·3%).

The α-cellulose was hydrolysed and the sugars were investigated by chromatograms and electrophoresis. Glucose, galactose, arabinose, mannose, xylose and rhamnose were identified. The colour of the spots of glucose and galactose was relatively much more intense than that of the other sugars. A hydrolysate of the hemicellulose contained galactose, mannose, arabinose, xylose and rhamnose. The colour of the galactose spot was relatively much more intense than that of the other sugars. A solution of the hemicellulose gave no colour with dilute iodine solution and contained no starch.

Separation of alkali-soluble and -insoluble polysaccharides from the whole cell

Freeze-dried *Chlorella* cells (50 g.) were extracted with 1·2 l. of methanol on a boiling-water bath for 6 hr. and filtered, and the pale-green residue was washed with methanol. The cells were then re-extracted with 600 ml. of boiling acetone for 6 hr. and filtered, and washed with acetone and dried. The cells, which were still coloured a light green, were treated with 500 ml. of 3% NaOH at 100° under N_2 for 6 hr. The residue was centrifuged and washed twice with water and then re-extracted at room temperature with 200 ml. of 24% KOH, centrifuged and washed six times with water. The residual material was dried. Yield, 1·05 g., 2·1% (N, 0·02%; ash, 0·8%).

The 3% NaOH extract and the washings were combined, cooled in ice and brought to pH 4·2 with acetic acid. A precipitate formed, which was centrifuged off. After evaporation to small bulk under reduced pressure, the supernatant liquid was poured into 2 l. of ethanol, whereupon a precipitate formed. This was separated by centrifuging, dried and dissolved in 130 ml. of water. The solution was cooled in ice and 100 ml. of trichloroacetic acid added in small portions until precipitation was complete. The mixture was centrifuged and the supernatant liquid was added to 2 l. of ethanol, whereupon a white flocculent precipitate of the soluble polysaccharides was obtained. This was collected, washed with ethanol and dried. Yield 4·34 g., 8·7% (N, 0·1%; ash, 0·05%).

Insoluble polysaccharides. These were hydrolysed with acid and then investigated by chromatography and electrophoresis, which showed the presence of glucose and galactose. This fraction probably corresponded to the α-cellulose isolated from the cell walls.

Soluble polysaccharides. A solution of this fraction gave a blue colour with dilute iodine solution. Acid hydrolysis followed by chromatography and electrophoresis showed the presence of galactose, glucose, arabinose, mannose, xylose and rhamnose. A solution of the polysaccharides (100 mg. in 10 ml. of borate buffer, pH 9·2) was investigated in a Tiselius electrophoresis apparatus (Perkin–Elmer and Co., U.S.A.; Model 38) by the method described by Northcote (1954). Three peaks were observed with mobilities of $2{\cdot}5 \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$, $7{\cdot}48 \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$ and $12{\cdot}5 \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$. Calculated from the areas under the curves the relative amounts of the components present were approximately 2:4:1 respectively.

The solution of the polysaccharides was investigated by zone electrophoresis on glass paper with borate buffer, pH 9·2 (Fuller & Northcote, 1956). Three spots were obtained. The distances of the spots from the starting line towards the cathode were 0·0, 3·0 and 5·0 cm.; 2:3:6-tri-*O*-methyl-D-glucose moved 13 cm. Starch used as a marker gave a spot at 5·0 cm. from the origin. The spot from the mixture which moved to 5·0 cm. was shown to be starch by its blue-staining reaction with iodine vapour. A solution of the hemicellulose isolated from the cell wall gave a spot at 3·0 cm. from the origin.

Purification of a soluble polysaccharide from the whole cell. The alkali-soluble polysaccharide (1 g.) was dissolved in 50 ml. of water to give a clear solution. To this was added a solution (2·5 ml.) containing amylase (prepared from human saliva by adding 10 vol. of water and centrifuging) and the mixture was kept at 25° for 30 hr. under toluene. A control solution containing 0·5 g. of soluble starch in 50 ml. was also treated in the same way and this, after incubation, gave a light-red colour with iodine solution. The solution of the polysaccharides from *Chlorella* was dialysed against running tap water for 12 hr. and then against 10 l. of distilled water for 6 hr. A light precipitate formed in the polysaccharide solution and this was removed by centrifuging. The clear supernatant was precipitated with 350 ml. of 80% (v/v) ethanol. The precipitate was collected and dried; yield, 0·4 g. A solution of this material gave no colour with iodine. In the Tiselius apparatus it gave two peaks with mobilities of $8{\cdot}7 \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$ and $12{\cdot}5 \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$; the relative amounts of the components present were approximately 3·5:1. Thus the peak with the mobility of $2{\cdot}5 \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$ in the original alkali-soluble polysaccharide fraction was identified as starch. Electrophoresis on glass paper showed one spot corresponding to the substance moving at 3·0 cm. in the original material.

A solution of 0·1 g. of the alkali-soluble polysaccharides in 2 ml. of borate buffer (pH 9·2) was put on a glass-powder electrophoresis column (Hocevar & Northcote, 1957) and a separation of the components obtained. A polysaccharide ($[\alpha]_D^{17} + 17{\cdot}1$ in water *c*, 1·0; *l*, 2·0) was obtained which gave one peak in the Tiselius electrophoresis apparatus with a mobility of $9{\cdot}9 \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$. This corresponded

with the component with a mobility of $7{\cdot}48 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$ in the original mixture or that with a mobility of $8{\cdot}7 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$ in the amylase-treated material. On glass-paper electrophoresis it gave one spot corresponding to the substance moving at 3·0 cm. in the original mixture and to the hemicellulose isolated from the cell wall. An examination of the acid hydrolysate of this polysaccharide showed the presence of galactose, arabinose, mannose, xylose and rhamnose. The colour of the galactose spot on the chromatogram was relatively much more intense than that of the other sugars.

Analyses of the cell walls treated with snail digestive enzyme

Cells walls (32·7 mg.) were incubated at 25° at pH 6·0 with snail digestive-enzyme preparation and control experiments were carried out concurrently with enzyme in the absence of cell walls and with cell walls in the absence of enzyme. After 14 hr., the cell walls were centrifuged, and washed with water and dried; yield, 25·8 mg. (loss of wt. 21·1%). No precipitate was observed in the incubation without cell walls and no loss in weight was apparent in the incubation without enzyme.

The digested walls were then analysed by the procedure already described. Found: lipid, 1·7 mg., 5·2%; hemicellulose, 8·8 mg., 27% (N, 0·5%; ash, 0·2%); α-cellulose 1·5 mg., 4·6% (N, 0·0%; ash, 0·2%). Compared with the original walls the material lost was made up of lipid, 4·0%, hemicellulose, 4·0%, and α-cellulose, 10·8%, which accounts for a total loss of material of 18·8%. Very little protein was removed, probably because of the weak proteolytic activity of the enzyme preparation. In terms of the individual constituents of the wall the digestion had removed 70% of the total α-cellulose, 43% of the lipid but only 13% of the hemicellulose.

Microscopical examination of the cell wall

The cell walls were examined by the optical and electron microscope. Whole cells can easily be distinguished under the optical microscope. Many fields of numerous preparations were examined and few whole cells were detected. The electron-microscope examinations showed that in nearly all cases few small cell particles contaminated the preparations.

A comprehensive examination of the cell wall was made under the electron microscope with a Sieman's Elminskop and with a high-tension voltage of 80 kv. Both unsectioned and sectioned material was examined. The preparations were supported on nitrocellulose films stabilized with carbon, which were mounted on Athene copper grids (New 200, diam. 2·3 mm.; Smethurst, High-Light Ltd., Bolton, Lancs.).

Unsectioned material was examined as follows. The walls were suspended in water and allowed to dry at room temperature in air on to the specimen grids. These were shadowed with uranium or chromium in an experimental evaporator (Coslett & Horne, 1955). The shadowing angle was 60°.

Sectioned material was prepared by fixing the walls in buffered (pH 7·3) osmium tetroxide soln. (1%, w/v) for 2 hr. (Palade, 1952); they were then washed with water and dehydrated with ethanol in the normal manner. The specimens were embedded in a mixture of 85% (v/v) of butyl methacrylate and 15% (v/v) of methyl methacrylate which was polymerized with 1·5% (w/v) of benzoyl peroxide at 50°. The sections were cut with glass knives in a microtome described by Porter & Blum (1953).

Unsectioned material. The microfibrils of the cell wall could be seen in the untreated preparations (Plate 1, Fig. 2), but were more apparent when the walls were treated with dilute NaOH soln. at room temperature (Plate 2, Fig. 3*a–e*). The NaOH soln. used was either 0·5 or 3% (w/v), and the time of exposure to the alkali varied from 5 min. to 24 hr. With the stronger solution and times of over 30 min. the wall lost its shape and eventually disintegrated into bundles of isolated fibres (Plate 1, Fig. 4). The milder treatment revealed the microfibrils as structures having a diameter of approx. 30–50 Å existing as a complete network over the wall and lying in two principal directions at right angles to one another (Plates 1 and 2, Figs. 5 and 3). In some of the specimens (Plate 1, Fig. 2) the inner surface of the wall could be seen at the characteristic cleavage which occurred when the cells were broken in the cell disintegrator. The orientation of the microfibrils in this region was the same as that on the outer surface. Generally the microfibrils were irregularly interwoven throughout the entire wall and covered the wall completely (Plates 1 and 2, Figs. 2 and 3). The microfibrils could also be exposed by treating the walls with ethanolamine (Wise, Peterson & Harlow, 1939) for 4 hr. at 25° (Plate 1, Fig. 5). The characteristics of these preparations were the same as those described above.

The intermicellar material was revealed as a granular substance packed around the microfibrils and could be seen *in situ* in the preparations which were treated with NaOH soln. for short times (Plate 2, Fig. 3). When the walls were treated with snail digestive enzyme (see above) they retained their shape but no microfibrils could be seen. Instead the surface of the wall appeared rough and made up of a granular substance (Plate 3, Fig. 6). The walls under these conditions were quite mechanically sound and could be centrifuged, placed on the supporting grids and embedded in the acrylate without disintegration. However, if these preparations were treated with 0·5% NaOH soln. for 5 min. or longer they completely fell apart and very little insoluble material could be recovered. Material previously treated with 0·5% NaOH soln. for 25 min. and then with the enzyme preparation still retained the shape of the cell wall and although very thin and fragile could be recovered. When examined microscopically it had a granular appearance (Plate 1, Fig. 7).

Sectioned material. The difficulty in the interpretation of the sectioned material was that of distinguishing definite layers in the wall from a layer-like appearance due to the representation by a two-dimensional photograph of a three-dimensional section in which the top and bottom edges of the section could appear as layers in the photograph. In some cases (Plate 4, Fig. 8) sections of the wall could easily be recognized as ribbon-like structures having a top and bottom edge. But generally our interpretation of the sectioned material is of necessity cautious and depends upon a close study of a very large number of such preparations.

The section of the whole cell (Plate 3, Fig. 9) showed that the preparation as isolated (Plate 4, Fig. 8) did represent the outer membranes of the cell. The thickness of the wall measured from these sections (Plates 3 and 4, Figs. 9 and 8)

was approx. 210 Å. The overall diameter of the cells was about 3–4 μ. In the section shown in Plate 3, Fig. 9, the outer wall appeared to consist of at least two membranes and after treatment with the digestive enzyme this division into membranes became much more apparent (Plate 4, Fig. 10). Each membrane indicated by the dark line, i.e. electron-dense or osmium-stained material, was approx. 50 Å thick and the space between them was approx. 100 Å. Thus the section at this region was in fact approx. 200 Å deep, which represents the thickness of the intact wall.

An interesting characteristic of these sections is the pronounced curling which takes place at the breakage cleft (Plate 4, Figs. 8 and 10). It was not ascertained at what stage after breakage this occurred.

The preparations treated with dilute NaOH soln. showed no definite lamellae but the walls seemed to have swollen (Plate 3, Fig. 11). The reticulate nature of the microfibrils was continuous throughout the section with no obvious lamellae due to local concentrations or variations in orientation. The microfibrils when stained with osmic acid appeared as a rather fluffy haze.

Sections of walls treated first with dilute NaOH soln. and then with the enzyme preparation were difficult to stain with osmic acid and showed no appearance of membranes.

DISCUSSION

The cell wall of *Chlorella pyrenoidosa* has been obtained by mechanical breakage and differential centrifuging. It is not contaminated by whole cells or cell debris. The material isolated has a fairly constant composition and when examined microscopically it resembles the wall of the intact cell. The cell wall has a thickness of approximately 210 Å whereas the diameter of the whole cell is 3–4 μ.

The general analysis of the cell wall shows an approximate composition of protein, 27 %; lipid, 9·2 %; α-cellulose, 15·4 %; hemicellulose, 31·0 %; glucosamine, 3·3 %; ash, 5·2 % (containing iron and calcium), which accounts for over 90 % of the material. The protein content of the wall is high compared with the few analyses that have been made on other plant-cell walls from soft tissues (Thimann & Bonner, 1933; Tripp & Rollins, 1952; Wirth, 1946). It has been calculated in this analysis from the nitrogen content of the walls after correcting for the amount of amino sugar present. This protein may be structural in function or metabolically active as part of the synthetic system of the cell-wall constituents, or both. The presence of the amino sugar is of interest in relation to the high protein content of the wall and might indicate the presence of a glycoprotein. It seems unlikely that an insoluble polysaccharide such as chitin is present since the glucosamine component is soluble in dilute sodium hydroxide.

No uronic acids were detected in the chemical analyses and if these are present they are presumably in very low concentrations. Normally the uronic acid constituents of the plant-cell wall are associated with the middle lamella or intercellular material which is absent in this unicellular alga.

The α-cellulose of the wall as isolated by the analytical procedure is composed of polysaccharide(s) made up of galactose, arabinose, mannose, xylose and rhamnose in addition to glucose.

The hemicellulose fraction of the cell wall on hydrolysis gives rise to galactose in relatively large amounts and also to mannose, arabinose, xylose and rhamnose. The alkali-soluble polysaccharides from the whole cells have been investigated electrophoretically and shown to contain at least three components. One of these has similar characteristics to that obtained from the cell wall and another is starch. An electrophoretically pure polysaccharide has been obtained from this mixture which resembles the material isolated from the wall. The purification was achieved and followed analytically by using electrophoretic procedures. The hemicellulose has a mobility of $9{\cdot}9 \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$ in 0·05 M-borate buffer, pH 9·2, 0° and $[\alpha]_D^{17}$ of $+17{\cdot}1°$.

The yield of hemicellulose and α-cellulose from the whole cell together with the analytical amounts of these constituents in the isolated wall enables the percentage (w/w) of wall in the whole cells to be calculated. If the α-cellulose yield from the whole cell is taken to have been 2·1 % then the cell wall represents 13·6 % (w/w) of the dry weight of the cell. Thus in the isolation procedure employed in this work about 28 % (w/w) of the total available wall material is isolated. The yield of walls was undoubtedly lowered by the differential centrifuging, as indicated in Fig. 1, but this loss is necessary if the preparation obtained is to be free from cell debris and whole cells.

The electron-microscope studies have shown the wall to consist of the usual two-phase system which is known to occur in the cell walls of most higher plants (Northcote, 1958). This consists of a microfibrillar structure embedded in a continuous matrix. The microfibrils have a diameter of 30–50 Å and are irregularly interwoven in a continuous network over the wall. Two main directions of the microfibrils are apparent, lying at right angles to one another. There seems to be no difference in the general arrangement and orientation of the microfibrils on the inner and outer surfaces of the wall and sections of wall treated with dilute sodium hydroxide solution showed no local concentrations of microfibrils. In these respects the wall resembles the primary cell wall of higher plants rather than the secondary wall. In some primary walls, however, differences in orientation of the microfibrils from the outside to the inside of the cell have been observed (Scott, Hamner, Baker & Bowler, 1956). Chemically the microfibrils correspond to the

isolated α-cellulose fraction and thus contain polysaccharides composed of monosaccharides other than glucose. It is possible that some of the material containing these other sugars may be adsorbed on to the microfibrils from the continuous matrix during their preparation. The matrix does contain a polysaccharide composed of these non-glucosidic sugars. However, treatment of the wall with 3% solutions of sodium hydroxide seems to give microfibrils which are free from the matrix substance when these are examined microscopically.

The matrix has a granular appearance and is continuous over the cell surface. Chemically it is related to the substances soluble in dilute sodium hydroxide. These are the hemicellulose, protein, amino sugar and possibly the lipid. The impure hemicellulose contains a high proportion of the nitrogen present in the original cell wall. The walls digested with the snail enzyme lose 70% of their α-cellulose, 43% of their lipid and only 13% of their hemicellulose. This accounts for nearly all the material digested by the enzyme, so that very little of the protein of the wall is removed. The material left shows no microfibrillar structure but resembles the granular matrix, and when sectioned these digested walls have a laminated appearance. Two distinct layers approximately 50 Å thick can be seen, one near the outer edge and one near the inner edge, separated by a space of 100 Å. This would indicate local concentrations of some of the materials of the matrix in these outer and inner lamellae although it is not implied that these matrix materials are completely localized in these regions. The sections of the wall show an interesting characteristic curling at the breakage cleft, and although several explanations of this are possible it might well indicate differences in molecular composition of the outside and inside regions of the cell wall.

SUMMARY

1. A cell-wall fraction of *Chlorella pyrenoidosa* has been isolated after disintegration of the whole cells in a Mickle cell disintegrator followed by differential centrifuging.

2. The isolated wall has been shown to be free of whole cells and debris. It is approximately 210 Å thick and represents 13·6% of the dry weight of the whole cell.

3. A quantitative chemical analysis of the wall has been made with respect to the following fractions: α-cellulose, hemicellulose, protein, lipid and ash. Glucosamine has been estimated.

4. The structure of the wall has been studied by the electron microscope and shown to be a two-phase system in which microfibrils approximately 30–50 Å in diameter are enclosed in a continuous matrix.

5. The protein, some of which may exist as a glycoprotein, is associated with the hemicellulose and with these polysaccharides makes up the greater part of the continuous matrix.

6. The microfibrillar structure, which is nearly all polysaccharide, corresponds to the α-cellulose of the chemical analysis. It is composed of polymers of glucose, galactose, mannose, arabinose and rhamnose.

7. The hemicellulose can be isolated as an electrophoretically pure polysaccharide from the whole cell and some of its characteristics have been studied quantitatively. An acid hydrolysate contains galactose, mannose, arabinose, xylose and rhamnose.

8. The microfibrils are present as a continuous irregular network over the cell wall and throughout its thickness. They lie approximately in two directions at right angles to one another.

9. The continuous matrix is granular in appearance. Microscopical examination and chemical analysis of cell walls treated with snail digestive enzyme show that some of the substances of which it is composed are partially localized in two regions. One of these is near the outer surface and the other near the inner surface of the wall.

One of us (R.W.H.) wishes to thank the Agricultural Research Council for financial aid. The Sieman's Elminskop was purchased by a generous financial grant from the Nuffield Foundation. We wish to thank Miss F. L. Myers for providing the enzyme preparation from *Helix pomatia*.

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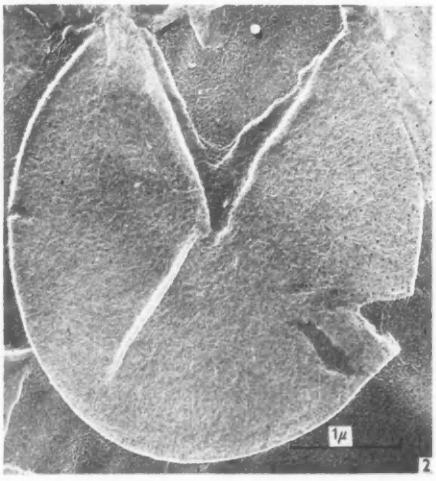
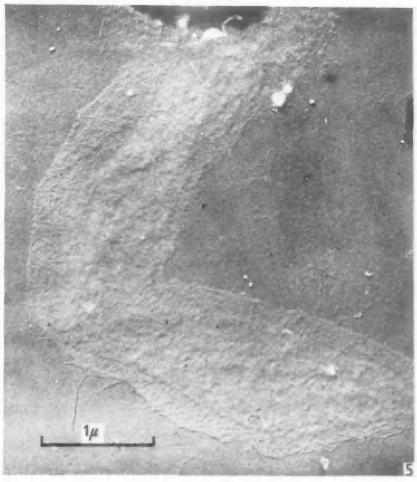
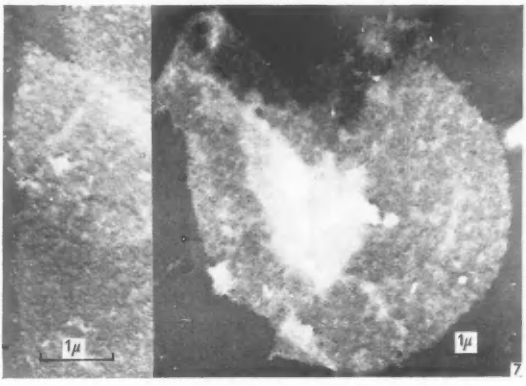
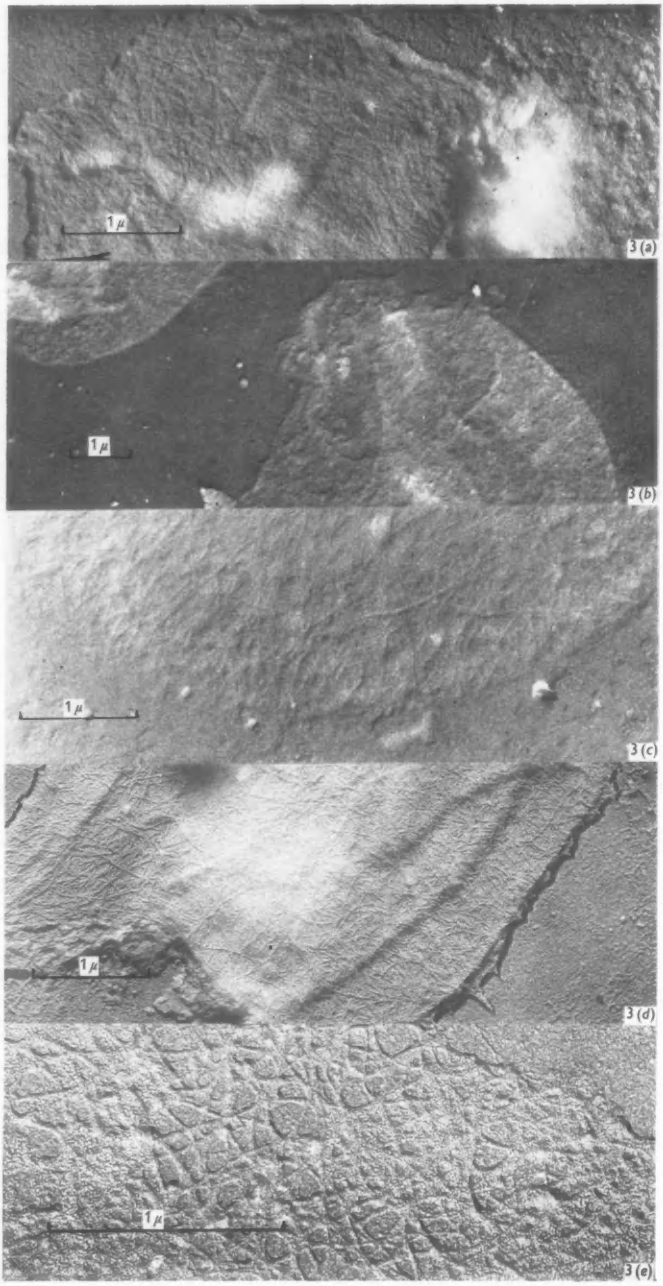






D. H. NORTHCOTE, K. J. GOULDING AND R. W. HORNE—THE CHEMICAL COMPOSITION AND STRUCTURE OF THE CELL WALL OF *CHLORELLA PYRENOIDOSA*

(*Facing p.* 396)



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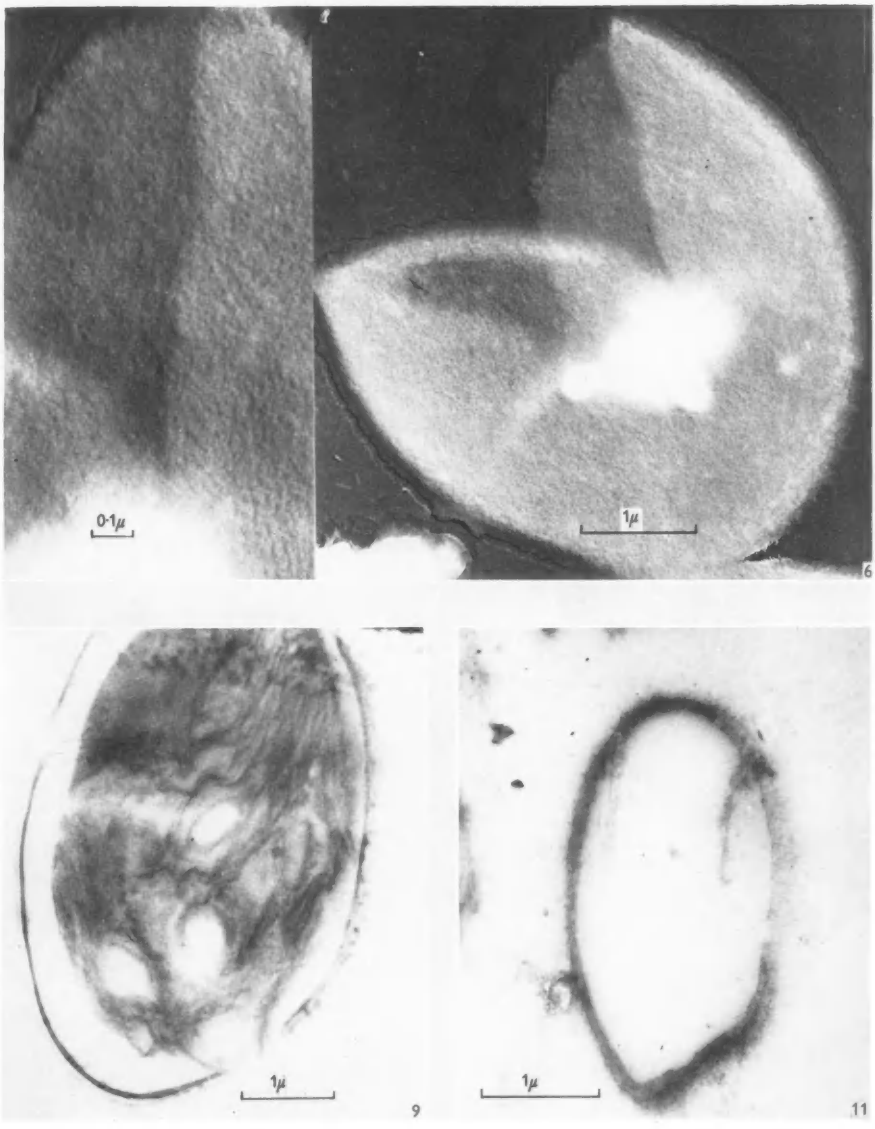

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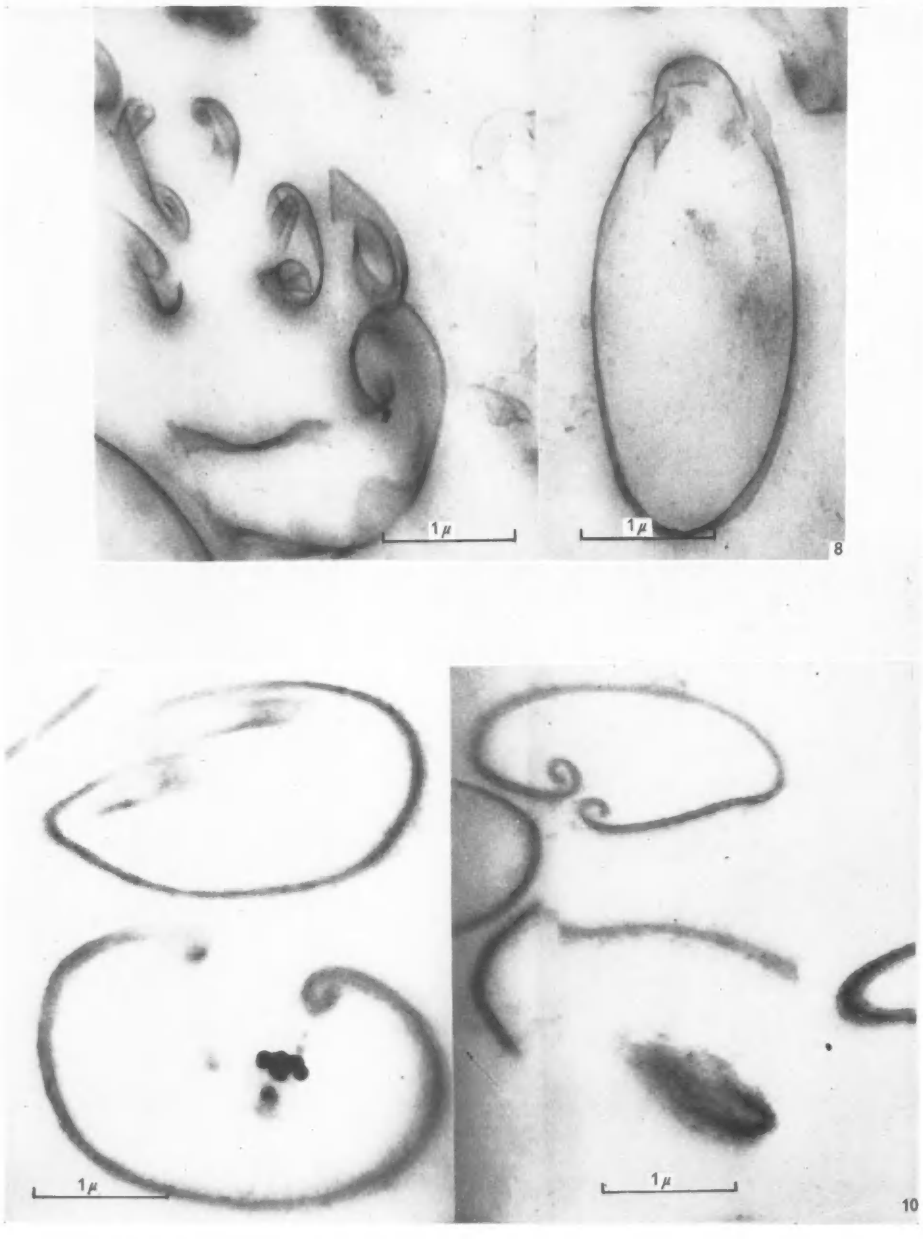

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EXPLANATION OF PLATES 1–4

Plate 1

Fig. 2. Untreated cell walls of *Chlorella*. Microfibrils can be seen and the undersurface of the wall is visible at the breakage cleft.

Fig. 4. Cell walls treated with 3% NaOH for 14 hr. at room temperature. The cell wall has completely disintegrated, giving bundles of microfibrils with very little matrix material visible.

Fig. 5. Cell walls treated with ethanolamine for 4 hr. at 25°, showing microfibrils.

Fig. 7. Cell walls treated with 0·5% NaOH for 25 min. at room temperature and then snail digestive enzyme at 25° for 14 hr. No microfibrils are visible and although the wall is very thin it still retains the cell shape.

Plate 2

Fig. 3. Cell walls treated with NaOH at room temperature: (*a*) 0·5% NaOH for 5 min.; (*b*) 0·5% NaOH for 25 min.; (*c*) 0·5% NaOH for 25 min.; (*d*) 0·5% NaOH for 30 min.; (*e*) 3% NaOH for 30 min. Microfibrils lying in two principal directions at right angles to one another can be clearly seen. The granular material of the matrix is visible. In (*e*) the wall has lost its form and is disintegrating into microfibrils.

Plate 3

Fig. 6. Cell walls treated with snail digestive enzyme for 14 hr. at 25°, showing rough granular surface and absence of microfibrils.

Fig. 9. Section of whole cell. The appearance of layers in the cell wall is visible. Compare with Plate 4, Fig. 8.

Fig. 11. Section of cell walls treated similarly to those shown in Plate 2, Fig. 3*c*. The walls have swollen and the microfibrils are visible as a diffuse material throughout the thickness of the sections.

Plate 4

Fig. 8. Sections of untreated cell walls showing ribbon-like appearance and characteristic curling at the breakage cleft. Compare with Plate 1, Fig. 2 and Plate 3, Fig. 9.

Fig. 10. Section of cell walls treated similarly to those shown in Plate 3, Fig. 6. The appearance of two layers in the wall is very apparent.

Phosphorolysis of Citrulline by Mammalian Liver: the Effect of a Bacterial Activator

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In a previous paper (Krebs, Eggleston & Knivett, 1955) it was shown that washed suspensions of *Escherichia coli* N.C.I.B. 8571 accelerate the phosphorolysis of citrulline by mammalian liver extracts. The bacterial cells contained ornithine decarboxylase, and most of the acceleration could be ascribed to the removal of ornithine, which acted as an inhibitor of the phosphorolysis. However, an accelerating effect remained even when the activity of ornithine decarboxylase was abolished by hydroxylamine. From this it was concluded that this strain of *E. coli* contained an additional substance (or 'factor') which promotes the phosphorolysis of citrulline.

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According to Jones, Spector & Lipmann (1955*a*) carbamyl phosphate is an intermediate in the synthesis of citrulline, and it may therefore be expected that the phosphorolysis of citrulline primarily leads to a formation of ornithine and carbamyl phosphate, and further that carbamyl phosphate, like ornithine, inhibits phosphorolysis. If this were the case, phosphorolysis would be accelerated by agents which promote the hydrolysis of carbamyl phosphate. This paper is concerned with the examination of the question whether the bacterial 'factor' is a carbamyl phosphatase.

EXPERIMENTAL

Carbamyl phosphate. This was prepared as the dilithium salt according to Jones, Spector & Lipmann (1955*b*). The salt was analysed for phosphate and NH_3 before and after complete hydrolysis in 0·01 N-HCl at 100° for 1 min. After correction for free phosphate (5%) and free NH_3 (1%) the purity of the salt was calculated to be of the order of 97–98%. Ammonia was determined by direct nesslerization. When the salt was added to H_2SO_4 solution (2%, v/v), the yield of CO_2, measured manometrically, was only 92% of the expected value. The reason for this low yield is obscure.

Buffers. ββ-Dimethylglutarate buffers were made according to Stafford, Watson & Rand (1955), 'tris maleate' buffers according to Gomori (1955), sodium phosphate buffers from mixtures of Na_2HPO_4 and NaH_2PO_4, citrate buffers from sodium citrate and NaOH. A glass electrode and a Pye pH meter were used to measure the pH of these buffers after dilution to the concentrations used in the experiments.

Liver preparations. Rat or guinea-pig liver was broken up in a Waring Blendor with 4 parts of water; this suspension was dialysed against ice–water for 24 hr. and stored at −15°. A purified preparation of the system responsible for the phosphorolysis of citrulline was made from an acetone-dried powder of guinea-pig liver, which was extracted with 25 vol. of water and subjected to $(NH_4)_2SO_4$ fractionation according to Krebs *et al.* (1955). The precipitate formed after 60% saturation was dissolved in water amounting to one-fifth of the volume of the supernatant and dialysed against ice–water for 24 hr. A precipitate formed during the dialysis was removed. The supernatant contained the enzyme system. It was stored at −15°; on thawing a precipitate appeared which was evenly suspended before pipetting the material.

Other animal tissues. One part of freshly obtained tissue, chilled in iced saline, was disintegrated in a stainless-steel Potter–Elvehjem homogenizer with 10 parts of ice–water.

Escherichia coli. The strains used and their growth have been described by Eggleston (1957).

Estimation of 'factor' activity. The 'factor' activity is defined as the increase in CO_2 evolution from citrulline in the presence of liver enzyme and phosphate, when both *E. coli* preparations and hydroxylamine are added. The CO_2 evolution was measured manometrically in a Warburg apparatus. The main compartment of the flasks usually contained 0·5 ml. of liver enzyme, 0·25–1·0 ml. of other enzyme preparations (*E. coli* or animal tissues), 0·5 ml. of M-sodium phosphate buffer, pH 6·8, and 0·25 ml. of 0·1 M-hydroxylamine-HCl. The side arm contained 0·5 ml. of 0·5 M-DL-citrulline and the gas space N_2. The total volume of fluid was 2·5–3·0 ml. The citrulline was added after thermal equilibration for 15 min. at 40°. In some experiments 0·5 ml. of 10% (v/v) H_2SO_4 was added from a second side arm at the end of the incubation period in order to release CO_2 bound by the buffer. Corrections were made for the small CO_2 evolution which occurred in controls containing *E. coli* and liver or *E. coli* and citrulline. Hydroxylamine was present in these controls.

Estimation of 'carbamylphosphatase' activity. The rate of decomposition of carbamyl phosphate was measured either by manometric determination of the rate of CO_2 evolution or by colorimetric phosphate determination. In the manometric test the side arm contained 0·2–0·4 ml. of 0·05 M-carbamyl phosphate, freshly dissolved, and the main compartment buffer and the enzyme preparation. The temperature was 25°. When the enzyme activity was measured by inorganic phosphate determination, the incubation was carried out in centrifuge tubes. The total volume was 1 ml. and the final concentrations of substrate and buffer the same as in the Warburg flasks. The reaction was stopped by the addition of 1·5 ml. of 5% (w/v) ice-cold trichloroacetic acid and the protein precipitate was immediately spun down at 0° for 3 min. A 1 ml. sample of the supernatant was removed for phosphate determination by the method of Berenblum & Chain (1938) or by the method of Lowry & Lopez (1946) as modified by Peel, Fox & Elsden (1955).

Table 1. *Relative 'factor' activities in fractions of* Escherichia coli *N.C.I.B.* 8571

Enzymic assay was carried out as described in the Experimental section. Q_{CO_2} = μl. of CO_2 evolved in 60 min./mg. dry wt.

No.	Fraction		Q_{CO_2}
	Cell suspension		4·6
	Cell-free extract		4·0
1	Ppt. from $MnCl_2$ addition, dialysed	insoluble fraction	0·6
		soluble fraction	8·8
2	Ppt. from 40% saturation of supernatant with $(NH_4)_2SO_4$, dialysed	insoluble fraction	1·2
		soluble fraction	0
3	Ppt. from 60% saturation of supernatant with $(NH_4)_2SO_4$, dialysed	insoluble fraction	4·7
		soluble fraction	6·1
4	Ppt. from 80% saturation of supernatant with $(NH_4)_2SO_4$, dialysed	insoluble fraction	6·8
		soluble fraction	60·0
5	Ppt. from final acidification with trichloroacetic acid to pH 2		5·7

Fractionation of 'factor' activity in Escherichia coli *N.C.I.B.* 8571. Thick, cream-like suspensions of *E. coli* cells from 5 l. of growth medium were crushed in the Hughes press (Hughes, 1951) at −15 to −25° and extracted with 0·15 M-NaCl at 2° for 24 hr. The volume of the NaCl solution was 50 times that of the dry weight of the crushed cells. Uncrushed cells, cell debris and undissolved material were centrifuged off at 24 000 *g* for 30 min. To the supernatant was added one-twentieth of a volume of M-$MnCl_2$ and the precipitate, consisting mainly of nucleoproteins, was removed by centrifuging. The supernatant was then subjected to $(NH_4)_2SO_4$ fractionation, starting with 40% saturation. The precipitates formed at 40, 60 and 80%, and after final acidification with trichloroacetic acid to pH 2, were suspended or dissolved in one-fifth of the volume of the corresponding supernatant and dialysed against ice–water for 48 hr. The fractions were then centrifuged and the soluble and insoluble sub-fractions tested for 'factor' activity. The results are given in Table 1. The most active fraction, that formed after 80% $(NH_4)_2SO_4$ saturation, was completely soluble in water, but during dialysis a precipitate was formed. This contained only a minor fraction of the activity. Although all stages of the fractionation were carried out below 5°, there was a loss of 60–85% of the original total activity. The most active extracts were stored at −15° for further use.

RESULTS

Properties of the 'factor' system. The 'factor' activity increased with the citrulline concentration over the range tested (20–100 mM), and was approximately proportional to the amount of *E. coli* preparation up to about 10 mg. dry wt. of cells/flask. With this quantity of bacteria and 0·5 ml. of the purified liver enzyme, 80 μl. of CO_2 was formed in 60 min. at a DL-citrulline concentration of 0·1 M. The rate of the reaction was initially constant at high concentrations of DL-citrulline (0·1 M) but later gradually fell, probably because of the accumulation of ornithine. The reaction continued for several hours, an indication of the relative stability of the enzyme systems.

The pH optimum for 'factor' activity was found to be near pH 6·2 in phosphate buffer (Table 2).

Table 2. *Effect of* pH *on 'factor' activity*

Cups contained 0·5 ml. of liver enzyme, 0·75 ml. of the soluble pool of 'fraction 4' (Table 1) (2 mg. of protein); 0·5 ml. of M-phosphate buffer; 0·25 ml. of 0·1 M-hydroxylamine; 0·5 ml. of 0·5 M-DL-citrulline; 0·5 ml. of 10% (v/v) H_2SO_4 (second side arm). Total vol. 3·0 ml.; temp. 40°. Gas phase N_2.

pH	CO_2 formed in 120 min. (μl.) Free	Bound	Total
5·9	160	0	160
6·2	175	3	178
6·5	131	8	139
6·8	123	16	139
7·1	85	20	105
7·4	35	12	47

The optimum for the liver enzyme causing arsenolytic or phosphorolytic cleavage of citrulline is about pH 6·7 (Krebs *et al.* 1955).

Carbamyl phosphate decomposition. There was a rapid decomposition of carbamyl phosphate when incubated with purified 'factor' preparations, the rate of liberation of phosphate being equal to that of CO_2 evolution. At 40° and below pH 6·0 there was also a rapid non-enzymic decomposition of carbamyl phosphate amounting during the first hour to 20–40% of the substance present. At higher pH values and 25° the non-enzymic decomposition was only a few per cent./hr. Variations of pH from about 4·5 to 7·0 did not affect the rates of enzymic hydrolysis; at pH 4·0, 8·2 and 9·2 the rates were 73, 73 and 43% respectively of the maximum. The final amounts of CO_2 evolved on enzymic hydrolysis were identical with that released by strong acid at 40°, namely, 92% of the expected value.

According to Jones *et al.* (1955*a*) the decomposition of carbamyl phosphate proceeds in two stages, with the intermediate formation of carbamic acid:

$$(1)\quad NH_2CO\cdot O\cdot PO_2H_2 \xrightarrow{H_2O} NH_2\cdot CO_2H + H_3PO_4$$

$$(2)\quad NH_2\cdot CO_2H \xrightarrow{H_2O} (NH_4)HCO_3.$$

In order to ascertain which of the two steps is catalysed by *E. coli*, carbamyl phosphate was hydrolysed to carbamate and phosphate before incubation by placing 0·25 ml. of 0·1 M-carbamyl phosphate and 0·5 ml. of 0·1 N-KOH in the side arm of a Warburg flask. The phosphate was liberated quantitatively during thermal equilibration for 10 min. at 40°; the carbamate formed was almost completely stable under these conditions. The excess of KOH was neutralized on mixing by 0·25 ml. of 0·1 N-HCl added to the buffer in the main compartment. The decomposition of the carbamate at pH 6·0 was found to be very rapid and was not affected by the addition of purified 'factor' preparations. Thus the enzymic action is on stage (1).

Activities of 'factor' and 'carbamylphosphatase' in other strains of Escherichia coli *and in rat tissues.* Table 3 compares the 'carbamylphosphatase' activity of various *E. coli* preparations and of rat tissues, with their 'factor' activity, calculated per mg. dry wt. 'Carbamylphosphatase' activity was found in all these materials, but 'factor' activity was absent from strains T and 4, and only slight in strains 86, F and 8114; strain 8571 had the highest 'factor' activity. In the rat tissues tested no 'factor' activity could be detected. There is thus no parallelism between the two activities. This result dismisses the assumption that the 'factor' is a 'carbamylphosphatase'.

Effects of various substances on 'factor' and 'carbamylphosphatase' activities. The phosphorolysis of citrulline in the presence of liver and *E. coli* is accelerated by increasing concentrations of phosphate (see Krebs *et al.* 1955, Table 10). Since phosphate stimulates the bacterial ornithine decarboxylase (Eggleston, 1957), the phosphate effect could be attributed to its effect on the decarboxylase, but experiments in which sufficient hydroxylamine was added to inhibit ornithine decarboxylase almost completely, showed that phosphate also stimulates 'factor' activity. Thus increasing the

Table 3. *'Factor' and 'carbamylphosphatase' activities in different strains of* Escherichia coli *and in rat tissues*

'Factor' activity was measured in phosphate buffer, pH 6·8, at 40°, 'carbamylphosphatase' activity in 0·05 M-dimethylglutarate buffer, pH 5·25, at 25° with 5 mM-carbamyl phosphate as substrate and a final volume of 4·0 ml. Q_{CO_2} = μl. of CO_2 liberated/mg. dry wt. of enzyme/hr., for the period 0–30 min.

Material tested	'Factor' activity (Q_{CO_2})	'Carbamylphosphatase' activity (Q_{CO_2})
E. coli F, washed cells	2·1	20·1
E. coli 4, washed cells	0	12·8
E. coli T, washed cells	0	10·6
E. coli N.C.T.C. 86, washed cells	1·0	31·8
E. coli N.C.I.B. 8114, washed cells	1·1	12·2
E. coli N.C.I.B. 8571, washed cells	5·2	29·5
E. coli N.C.I.B. 8571, purified extract from 80% $(NH_4)_2SO_4$ fractionation	60	270
Rat-lung homogenate	—	1·6
Rat-liver homogenate	0	5·7
Rat-kidney homogenate	0	5·8
Rat-spleen homogenate	0	13·3
Rat-brain homogenate	—	2·5
Rat-heart homogenate	—	1·9

Table 4. *Effect of* L-*asparagine,* L-*aspartate and fumarate on 'factor' activity*

Each cup contained 0·5 ml. of dialysed guinea-pig liver suspension; 0·5 ml. of *Escherichia coli* 8571 suspension (30 mg. dry wt.); 0·5 ml. of M-sodium phosphate buffer, pH 6·8; 0·3 ml. of 0·1 M-$NH_2OH \cdot HCl$; 0·5 ml. of 0·5 M-DL-citrulline. Final concentrations of L-asparagine, L-aspartate and fumarate were 33 mM. Total volume 3·0 ml. Gas phase N_2. Temp. 40°. Results represent μl. of CO_2 liberated.

Additions	Period 1 (0–60 min.)	Factor activity	Period 2 (60–120 min.)	Factor activity	Period 3 (120–240 min.)	Factor activity
None	34	—	22	—	44	—
Citrulline	88	54	54	32	85	41
Asparagine	36	—	40	—	173	—
Asparagine + citrulline	86	50	60	20	269	96
Aspartate	37	—	55	—	171	—
Aspartate + citrulline	100	63	101	46	375	204
Fumarate	68	—	110	—	197	—
Fumarate + citrulline	122	54	211	101	359	162

Table 5. *Effect of pre-incubation with aspartate or fumarate on the production of an increased 'factor' activity*

Conditions were as described in Table 4, except that final concentrations of L-aspartate and fumarate were 28 mM. Total vol. 3·6 ml. in N_2 at 40°. Shaken for 2 hr. before addition of citrulline from the side arm. Results represent μl. of CO_2 liberated after the addition of citrulline.

Cup no. ...	1	2		3	4		5	6	
Additions ...	None	Citrulline	Factor activity (2 − 1)	Aspartate	Aspartate + citrulline	Factor activity (4 − 3)	Fumarate	Fumarate + citrulline	Factor activity (6 − 5)
First 30 min.	19	61	42	142	258	116	181	293	112
Second 30 min.	22	59	37	105	263	158	191	299	108
Third 30 min.	28	49	21	74	212	138	96	219	123
Fourth 30 min.	32	63	31	51	165	114	61	170	109
Fifth 30 min.	32	71	39	50	161	111	54	158	104
Sixth 30 min.	33	73	40	46	128	82	44	111	67

Table 6. *Effect of adenosine 5′-phosphate (AMP) on 'factor' activity*

Conditions were as described in Table 4 except that dialysed rat liver was used and AMP was 10 mM final concentration where added. Controls (not shown) with AMP added, but omitting either liver or *E. coli*, gave no extra CO_2 in the presence of citrulline. Results represent μl. of CO_2 liberated.

Cup no. ...	1	2		3	4		
Additions ...	None	Citrulline	Factor activity (2 – 1)	AMP	AMP + citrulline	Factor activity (4 – 3)	AMP effect
First 30 min.	23	64	+41	103	152	+ 49	+ 8
Second 30 min.	34	67	+33	134	315	+181	+148
Third 30 min.	34	57	+23	269	464	+195	+172
Fourth 30 min.	39	63	+24	317	525	+208	+184

phosphate concentration from 16 mM to 0·2 M caused a threefold increase in the amounts of CO_2 liberated.

Adenosine 5′-phosphate (AMP), L-aspartate, L-asparagine, fumarate and glycerol were also found to increase the rate of 'factor' activity. The effects are not immediate but develop with time (Tables 4–6); the highest increases were obtained with AMP, where it was +20% after 30 min. and +420% after 2 hr. These substances had no effect on the hydrolysis of carbamyl phosphate. They all also increased the rate of CO_2 evolution in the absence of citrulline, because of the fermentation of the added substrate by *E. coli*. Citrulline has no effect on the rate of these fermentations, as measured by CO_2 production. The four named substances do not form CO_2 in the presence of the liver preparation without *E. coli*. It should be mentioned that the effects of aspartate, asparagine and fumarate were observed with sheep and guinea-pig liver, but not with rat liver; on the other hand, rat liver gave effects with glycerol and AMP.

Succinate (20 mM) and L-malate (17 mM) caused only small increases in 'factor' activity (15–30% in 2 hr.). Adenosine triphosphate (10 mM), yeast adenylic acid (10 mM), pyridoxal phosphate (10 μg./ml.), tetraethyl pyrophosphate (10 mM) and methanol (33 mM) were without effect.

Citrate (17 mM), pyruvate (17 mM), L-glutamate (17 mM), NH_4Cl (20 mM) and carbamyl phosphate (10 mM) inhibited 'factor' activity by 100, 66, 100, 84 and 52% respectively (incubation time 120–180 min.). The inhibition by carbamyl phosphate (which underwent rapid decomposition) may have been due to the NH_3 formed. The enzymic breakdown of carbamyl phosphate by *E. coli* was not inhibited by citrulline (33 mM).

DISCUSSION

The experiments reported in this paper show that *Escherichia coli* preparations which promote the phosphorolysis of citrulline by mammalian liver extracts in the presence of hydroxylamine contain a carbamylphosphatase, but there was no parallelism between the promotion of citrulline phosphorolysis and carbamylphosphatase activity. The factor in *E. coli* which, in addition to ornithine decarboxylase, promotes citrulline phosphorolysis, is thus unlikely to be a carbamylphosphatase. This conclusion is borne out by the fact that carbamylphosphatase preparations from animal tissues cannot replace the *E. coli* 'factor'. The nature of this 'factor' remains obscure.

The activity of the 'factor' is increased by aspartate, fumarate, adenosine 5′-phosphate and other substances, whereas the hydrolysis of carbamyl phosphate is not affected. This confirms the above conclusion.

The experiments also show that carbamyl phosphate is readily hydrolysed by enzymes present in animal tissues and in various strains of *E. coli*. Whether this hydrolysis is due to a specific enzyme or to one of the unspecific phosphatases has not been examined.

The possibility that carbamyl phosphate is an intermediate in the phosphorolysis of citrulline is not excluded by the present experiments.

SUMMARY

1. A heat-labile bacterial 'factor' (other than ornithine decarboxylase) capable of stimulating the phosphorolysis of citrulline by mammalian liver has been purified 15-fold by ammonium sulphate fractionation. The purified preparation and washed suspensions of several strains of *Escherichia coli* were tested for 'factor' activity and carbamyl phosphatase activity, because acceleration of the hydrolysis of carbamyl phosphate might account for the 'factor' activity.

2. Carbamylphosphatase activity was found in all 'factor' preparations, but there was no parallelism with 'factor' activity. The latter was absent from rat spleen, kidney and liver, although these tissues rapidly hydrolysed carbamyl phosphate, and *E. coli* N.C.I.B. 8571 was the only one of six strains which had appreciable 'factor' activity.

3. Adenosine 5′-phosphate, L-aspartate, L-asparagine, fumarate and glycerol each stimulated 'factor' activity but had no effect on the enzymic hydrolysis of carbamyl phosphate. This stimulation generally increased with time.

4. Some characteristics of the bacterial hydrolysis of carbamyl phosphate are described.

5. The results indicate that the bacterial 'factor' is not a carbamylphosphatase.

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The Formation of Acetoacetate in Homogenates of the Mammary Gland

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Apart from the liver, which is generally considered the principal site of ketogenesis (see Weinhouse, 1952; Campbell & Best, 1956), the rumen is also recognized as a source of the ketone bodies circulating in the blood of ruminants (see Shaw, 1956).

Mammary tissue, which possesses high lipogenic activity (Balmain, Folley & Glascock, 1952) and which is able to oxidize acetoacetate (Terner, 1955), must synthesize acetoacetyl-coenzyme A as an intermediate; this may give rise to ketone bodies. As reported in previous papers (Terner, 1954, 1956*b*), the addition of fumarate in increasing amounts to respiring mammary homogenates metabolizing ^{14}C-labelled acetate or pyruvate resulted in a progressive increase in the rate of fatty acid synthesis. In continuation of this work it was found that, when the rate of fatty acid formation was limited by decreasing the amount of added fumarate, acetoacetate was formed from acetate or pyruvate. In contrast with the powerful ketogenicity of pyruvate and acetate, glucose and lactate displayed only weak ketogenic activity. This analogous metabolic behaviour of glucose and lactate offers an explanation for the differences in ketogenic activity of the various substrates in terms of their ability to maintain an adequate level of reduced pyridine nucleotides in an actively oxidative environment and suggests a mechanism for the well-known antiketogenic properties of glucose. The results further indicate that the lactating mammary gland may deserve consideration as a site of ketone-body production in dairy animals suffering from ketosis. A preliminary account of part of this work has been given (Terner, 1956*c*, 1957).

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EXPERIMENTAL

The material, reaction mixtures, reagents and methods were the same as described in the earlier paper on lipogenesis in guinea-pig mammary homogenates (Terner, 1956*b*). Whole homogenates of mammary tissue were used; kidney-cortex homogenates were centrifuged at low speed for a few minutes to remove broken cells and nuclei.

Determination of acetoacetate. This was determined manometrically by the aniline method (Ostern, 1933; Edson, 1935) as modified by Krebs & Eggleston (1945). When radioactive substrates were employed, the 'bound CO_2' was first liberated from the medium by the addition of 1 ml. of a mixture of equal volumes of 50% citric acid and 3N-perchloric acid. The CO_2 was absorbed during the next 30 min. period in the NaOH in the centre wells of manometer flasks which had served to absorb the CO_2 during the measurements of O_2 consumption. The contents of the centre wells were transferred to a few millilitres of water in test tubes. The centre wells were washed with dil. HCl and charged with fresh portions (0·2 ml.) of 2N-NaOH and a strip of filter paper. The acetoacetate was then decarboxylated by adding 1 ml. of aniline citrate from the side arms of the vessels. After the reaction was complete, the contents of the centre wells were transferred to a second set of test tubes. Since aniline will also decarboxylate oxaloacetate, treatment with $Al_2(SO_4)_3$ (Krebs & Eggleston, 1945) was in some experiments interposed between the collection of the respiratory CO_2 and the addition of

aniline citrate. No radioactive CO_2 was liberated by the treatment with $Al_2(SO_4)_3$.

Determination of radioactivity. Carrier K_2CO_3 was added to all test tubes containing the NaOH from the centre wells and the carbonate precipitated with $BaCl_2$. The $BaCO_3$ was washed, dried, weighed and plated on planchets of 2·0 or 4·9 cm.² area. In earlier experiments 'infinitely thick' plates were prepared and counted with an end-window counter. The counting rates of thin samples prepared in later work were determined with a gas-flow counter and corrected for self-absorption. The specific activity of the radioactive substrates was determined by wet combustion of the material and counting as $BaCO_3$. The results were calculated in terms of amount of substrate appearing in the products. In calculating the amount of randomly labelled glucose oxidized to CO_2 and water, a correction was applied for the appearance in the respiratory CO_2 of C-3 and C-4 of that fraction of glucose which gave rise to ^{14}C-labelled acetoacetate. Although the radioactivity of only the terminal carboxyl group of acetoacetate was determined, it was assumed that both halves of the molecule were equally labelled and the radioactive acetoacetate was calculated as being derived from two molecules of radioactive pyruvate, lactate or acetate, and from one molecule of randomly labelled glucose respectively.

All labelled compounds were obtained from the Radiochemical Centre, Amersham.

Units. Respiratory rates are expressed as Q_{O_2} (μl./mg. dry wt./hr.). $Q_{ac.-ac.}$ denotes the rate of change in acetoacetate content of the reaction mixture as determined by the manometric method (1 μmole of acetoacetate = 22·4 μl.). Data derived from measurements of radioactivity are expressed as μm-moles/100 mg. dry wt./hr. The dry weights of the homogenates were determined by evaporation in a steam oven and were corrected for the salt content of the diluting fluid.

RESULTS

Acetoacetate formation and fatty acid synthesis in mammary homogenates. Experiments reported in this paper reveal an inverse relationship between the rate of acetoacetate formation by mammary homogenates and fumarate concentration. As shown in Table 1, the amount of pyruvate carbon appearing in acetoacetate decreased progressively with the increase in fumarate concentration. The effect of fumarate on fatty acid and acetoacetate formation from acetate is shown in Table 2. In addition to the isotopic data, a small but definite net increase in the amount of acetoacetate in the absence of fumarate, determined by the manometric technique, indicated a net synthesis of the ketone body.

Effect of inhibitors on acetoacetate formation. Since, even in the absence of added fumarate, substrate carbon appeared in the respiratory CO_2, the Krebs cycle was functioning, if only to a limited extent. Previous studies of the effects of various inhibitors on aerobic phosphorylation and lipogenesis in mammary homogenates (Terner, 1956*a*, *b*) were therefore extended to include their effects on the formation of acetoacetate. The addition of

Table 1. *Effect of fumarate on formation of acetoacetate from pyruvate*

Mammary homogenate (dry wt. 42·5 mg.), basal medium, [2-^{14}C]pyruvate (0·01 M) and acetoacetate (0·01 M) in all flasks. Incubation period was 75 min.

Additions	$-Q_{O_2}$	Pyruvate (μm-moles/100 mg. dry wt./hr.) appearing in: Respiratory CO_2	Pyruvate (μm-moles/100 mg. dry wt./hr.) appearing in: Acetoacetate
None	7·9	4800	4350
Fumarate, 1 mM	7·2	2160	1380
Fumarate, 2 mM	6·5	1060	1180
Fumarate, 5 mM	6·5	470	320
Fumarate, 10 mM	6·6	320	210

p-nitrophenol (0·2 mM) strongly depressed the incorporation of acetate carbon into acetoacetate, but retarded to a lesser extent its appearance in the respiratory CO_2 (Table 2). When [2-^{14}C]pyruvate was the substrate, the inhibitory action of *p*-nitrophenol on the rate of labelling of acetoacetate was relatively small, the radioactivity of the respiratory CO_2 being reduced somewhat more. The addition of fluoride (20 mM) also inhibited the appearance of pyruvate carbon in the respiratory CO_2 to a greater extent than its entry into acetoacetate. Arsenate (5–25 mM), especially when added in high concentration, diminished the formation of acetoacetate from pyruvate (Table 3).

Effect of the presence of carrier acetoacetate on metabolism of mammary homogenates. In order to determine the extent of dilution of [^{14}C]acetyl-coenzyme A, derived from the labelled substrates, by the acetyl-coenzyme A arising from the enzymic breakdown of initially unlabelled carrier acetoacetate, experiments were carried out in which all experimental conditions were duplicated, except that the carrier was either added or omitted. As shown in Table 4, the respiratory CO_2 contained more labelled carbon in the absence of the carrier than in its presence, as may be expected if, in the former case, some of the undiluted [^{14}C]acetoacetate had been broken down either enzymically during the incubation period, or spontaneously during the incubation period and during the subsequent period allowed for the absorption of the 'bound CO_2' (see Experimental). In the presence of fumarate, the radioactivity of the fatty acids and of the respiratory CO_2 was only slightly diminished by the presence of acetoacetate and only a small amount of the substrate carbon was trapped in the carrier.

Effect of octanoate and glucose. For the study of the effect on the present system of precursors of acetyl-coenzyme A of known ketogenic and antiketogenic properties, octanoate and glucose were

chosen. The addition of unlabelled octanoate to mammary homogenates metabolizing [*carboxy*-^{14}C]-acetate in the absence of fumarate resulted in a marked diminution, presumably by isotope dilution, of the radioactivity of the acetoacetate formed, accompanied by a much smaller decrease in the radioactivity of the respiratory CO_2 (Table 5). This finding is consistent with the ketogenic property of the fatty acid. The effect of glucose, however, was variable. In many preparations the addition of unlabelled glucose had almost no effect on the rate of appearance of [*carboxy*-^{14}C]-acetate carbon in acetoacetate, although the activity of the respiratory CO_2 was depressed.

Table 2. *Synthesis of fatty acids and acetoacetate from acetate*

Mammary homogenate (dry wt. 37·4 mg.), basal medium, [*carboxy*-^{14}C]acetate (5 mM) and acetoacetate (5 mM) in all flasks. Fumarate, 5 mM. Incubation period was 50 min.

Additions	$-Q_{O_2}$	$Q_{ac.-ac.}$	Acetate (μm-moles/100 mg. dry wt./hr.) appearing in: Respiratory CO_2	Acetoacetate	Fatty acids
None	7·3	+0·2	6880	2930	58
p-Nitrophenol, 0·2 mM	5·5	−0·4	4460	186	48
Fumarate	7·2	−1·4	2210	250	1060
Fumarate; *p*-nitrophenol, 0·2 mM	5·7	−0·5	1365	71	173
Fumarate; *p*-nitrophenol, 0·4 mM	3·8	−0·3	404	45	0

Table 3. *Effect of inhibitors on formation of acetoacetate from pyruvate*

Mammary homogenate (dry wt. 62 mg.), basal medium, [2-^{14}C]pyruvate (7·5 mM) and acetoacetate (10 mM) in all flasks. Fluoride, 20 mM. Incubation period was 65 min.

Additions	$-Q_{O_2}$	Pyruvate (μm-moles/100 mg. dry wt./hr.) appearing in: Respiratory CO_2	Acetoacetate
None	7·0	2940	3900
p-Nitrophenol, 0·2 mM	4·0	1280	2500
Fluoride	4·2	1030	3100
Arsenate, 5 mM	5·1	2280	2320
Arsenate, 10 mM	5·0	2060	2760
Arsenate, 25 mM	3·8	970	1800

Table 4. *Effect of carrier acetoacetate on metabolism of acetate*

Mammary homogenate (dry wt. 52·2 mg.), basal medium and [*carboxy*- ^{14}C]acetate (5 mM) in all flasks. Additions: acetoacetate, 5 mM; fumarate, 5 mM. Incubation period was 55 min.

Additions	$-Q_{O_2}$	Acetate (μm-moles/100 mg. dry wt./hr.) appearing in: Respiratory CO_2	Acetoacetate	Fatty acids
None	7·0	6600	—	60
Acetoacetate	7·6	4260	3200	20
Fumarate	9·0	1510	—	1840
Acetoacetate, fumarate	8·8	1090	150	1560

Table 5. *Fatty acid oxidation and acetoacetate formation*

Mammary homogenate (dry wt. 52 mg.), basal medium, [*carboxy*-^{14}C]acetate (7·5 mM) and acetoacetate (10 mM) in all flasks. Octanoate, 2·5 mM. Incubation period was 60 min.

Additions	$-Q_{O_2}$	Acetate (μm-moles/100 mg. dry wt./hr.) appearing in: Respiratory CO_2	Acetoacetate
None	6·7	5330	2280
Octanoate	6·7	4620	1090
Fumarate, mM	7·4	3930	1420
Fumarate, mM; octanoate	8·5	3590	534
Fumarate, 2 mM	8·2	2510	442
Fumarate, 2 mM; octanoate	9·0	2160	411
Fumarate, 5 mM	8·4	1245	250
Fumarate, 5 mM; octanoate	9·9	1230	169

When the rate of glucose breakdown was accelerated by the addition of hexokinase, the radioactivity of both the respiratory CO_2 and acetoacetate was diminished (Table 6), as might be expected to be the result of isotope dilution of the [^{14}C]acetyl-coenzyme A. In homogenates in which the rate of acetoacetate formation was slow in relation to the rate of $^{14}CO_2$ production, glucose increased the rate of incorporation of [*carboxy*-^{14}C]-acetate carbon into both the respiratory CO_2 and acetoacetate (Table 7). In the presence of glucose, the addition of *p*-nitrophenol inhibited the formation of acetoacetate from [*carboxy*-^{14}C]acetate to a much greater extent than it depressed the appearance of ^{14}C in the respiratory CO_2 (Table 8).

Glucose, pyruvate, lactate and acetate as precursors of acetoacetate. A comparison of the rates of appearance in acetoacetate of ^{14}C from labelled glucose, pyruvate and acetate showed that pyruvate and acetate were readily converted into acetoacetate. In many experiments acetate entered acetoacetate as rapidly as it appeared in the respiratory CO_2. Pyruvate appeared to show even stronger ketogenic tendencies, the amount of its carbon recovered from the ketone body often exceeding the amount oxidized to CO_2. The opposite was observed when [^{14}C]glucose (randomly labelled) was the only added substrate; only a small part of its carbon was found in acetoacetate, and by far the larger part appeared in the respiratory CO_2. It appears from Table 10 that glucose may undergo oxidation to CO_2 more readily than the other substrates examined. This may, however, be due to the contribution of C-3 and C-4 of that part of [^{14}C]glucose that may have been converted into fatty acids and for which no correction was applied. The low rate of conversion of glucose into acetoacetate was only slightly, if at all, increased

Table 6. *Effect of glucose and hexokinase on formation of acetoacetate from acetate*

Mammary homogenate (dry wt. 27·8 mg.), basal medium, [*carboxy*-^{14}C]acetate (4 mM) and acetoacetate (10 mM) in all flasks. Glucose, 20 mM. Incubation period was 60 min.

Additions	$-Q_{O_2}$	Acetate (μm-moles/100 mg. dry wt./hr.) appearing in	
		Respiratory CO_2	Acetoacetate
None	8·4	4140	2470
Glucose	8·3	2700	2430
Glucose; hexokinase, 4·5 units	11·2	2720	2360
Glucose; hexokinase, 9 units	10·1	1990	1540
Glucose; hexokinase, 18 units	9·5	1070	830

Table 7. *Stimulation by glucose of acetoacetate formation from acetate*

Mammary homogenate (dry wt. 23·5 mg.), basal medium, [*carboxy*-^{14}C]acetate (5 mM) and acetoacetate (10 mM) in all flasks. Glucose, 20 mM. Incubation period was 85 min.

Additions	$-Q_{O_2}$	Acetate (μm-moles/100 mg. dry wt./hr.) appearing in	
		Respiratory CO_2	Acetoacetate
None	4·0	2340	262
Glucose	8·0	3660	1520
Fumarate, 1 mM	7·5	3760	274
Fumarate, 1 mM; glucose	11·0	3660	1130
Fumarate, 2 mM	7·8	2140	156
Fumarate, 2 mM; glucose	9·2	1870	223

Table 8. *Effect of glucose and* p*-nitrophenol on formation of acetoacetate from acetate*

Mammary homogenate (dry wt. 59 mg.), basal medium, [*carboxy*-^{14}C]acetate (5 mM) and acetoacetate (10 mM) in all flasks. Glucose, 5 mM. Hexokinase, 18 units. Incubation period was 60 min.

Additions	$-Q_{O_2}$	Acetate (μm-moles/100 mg. dry wt./hr.) appearing in	
		Respiratory CO_2	Acetoacetate
None	6·5	3650	1825
Glucose	7·3	2900	2670
Glucose; *p*-nitrophenol, 0·2 mM	6·0	1650	278
Glucose; hexokinase	7·6	2540	1615
Glucose; hexokinase, *p*-nitrophenol, 0·2 mM	7·4	1860	410
Glucose; hexokinase, *p*-nitrophenol, 0·4 mM	5·6	1590	186

Table 9. *Formation of acetoacetate from glucose and acetate*

Mammary homogenate (dry wt. 34 mg.), basal medium and acetoacetate (10 mM) in all flasks. Additions: [^{14}C]glucose (randomly labelled), 5 mM; unlabelled glucose, 5 mM; [*carboxy*-^{14}C]acetate, 5 mM; hexokinase, 18 units. Incubation period was 60 min.

Additions	$-Q_{O_2}$	Labelled substrate (acetyl-coenzyme A equivalents) (μm-moles/100 mg. dry wt./hr.) appearing in: Respiratory CO_2	Acetoacetate
[^{14}C]Glucose	8·4	2135	585
[^{14}C]Glucose, hexokinase	10·3	4570	870
[^{14}C]Acetate	6·8	2040	2200
[^{14}C]Acetate, glucose	8·0	785	2220
[^{14}C]Acetate, glucose, hexokinase	9·4	756	1925

Table 10. *Formation of acetoacetate from pyruvate, lactate and glucose*

Mammary homogenate (dry wt. 15·8 mg.), basal medium and acetoacetate (10 mM) in all flasks. Additions: [2-^{14}C]-pyruvate, 5 mM; [2-^{14}C]DL-lactate, 5 mM; [^{14}C]glucose (randomly labelled), 5 mM; hexokinase, 18 units; *p*-nitrophenol, 0·2 mM. Incubation period was 80 min.

Additions	$-Q_{O_2}$	Labelled substrate (acetyl-coenzyme A equivalents) (μm-moles/100 mg. dry wt./hr.) appearing in: Respiratory CO_2	Acetoacetate
[2-^{14}C]Pyruvate	8·3	3010	5320
[2-^{14}C]Pyruvate, *p*-nitrophenol	5·4	2190	4440
[2-^{14}C]Lactate	5·9	3580	812
[2-^{14}C]Lactate, *p*-nitrophenol	4·5	2380	332
[^{14}C]Glucose	8·8	4300	700
[^{14}C]Glucose, *p*-nitrophenol	8·1	5300	590
[^{14}C]Glucose, hexokinase	10·2	6000	780
[^{14}C]Glucose, hexokinase, *p*-nitrophenol	10·2	8200	710

Table 11. *Acetoacetate formation by kidney cortex*

Rabbit kidney-cortex homogenate (dry wt. 19·8 mg.), basal medium and acetoacetate (10 mM) in all flasks. Additions: [^{14}C]glucose (randomly labelled), 5 mM; [2-^{14}C]-pyruvate, 5 mM; [2-^{14}C]DL-lactate, 10 mM; [*carboxy*-^{14}C]-acetate, 5 mM. Incubation period was 50 min.

Additions	$-Q_{O_2}$	Labelled substrate (acetyl-coenzyme A equivalents) (μm-moles/100 mg. dry wt./hr.) appearing in: Respiratory CO_2	Acetoacetate
[^{14}C]Glucose	20·0	13 280	2 090
[2-^{14}C]Pyruvate	15·9	4 770	11 160
[2-^{14}C]Lactate	17·0	7 920	1 430
[1-^{14}C]Acetate	17·6	8 620	9 680

by the addition of hexokinase, or of *p*-nitrophenol, or both, although these agents markedly accelerated the rate of $^{14}CO_2$ formation from glucose (Tables 9 and 10). In marked contrast with the powerful ketogenic properties of pyruvate and acetate, [2-^{14}C]lactate gave rise to only a small amount of acetoacetate, in a pattern strongly resembling that of glucose (Table 10). Similar observations were made when homogenates of rabbit-kidney cortex were substituted for mammary homogenates (Table 11).

DISCUSSION

Effect of fumarate on the pathways of acetyl-coenzyme A in mammary homogenates. The appearance of radioactive-substrate carbon in the respiratory carbon dioxide shows that even in the absence of added fumarate the Krebs cycle was operative and supplied sufficient energy for the activation of acetate. Endogenous precursors of oxaloacetate were present in the preparation in only low concentration, as evidenced by the marked effect of fumarate when added in catalytic amounts (1 mM). Further increases in the concentration of added fumarate (up to 5–10 mM) resulted in the progressive stimulation of fatty acid synthesis (Terner, 1956*b*) and at the same time in the suppression of acetoacetate formation. The progressive decline in the radioactivity of the respiratory carbon dioxide caused by increasing amounts of fumarate has been discussed before as due to isotope dilution of the C_4 dicarboxylic acids derived from the radioactive substrate when passing

through the fumarate stage of the cycle (Terner, 1956*b*), and should therefore not be regarded as indicating an inhibition of the oxidative pathway.

Exchange of acetyl-coenzyme A or synthesis of acetoacetate. Since acetoacetate is broken down by mammary homogenates (see Table 2), the possibility must be considered that the appearance of labelled acetoacetate may have been due entirely or in part to an enzymic-exchange reaction between the labelled acetyl-coenzyme A derived from the substrate and the unlabelled acetoacetyl-coenzyme A produced by the activation of the carrier acetoacetate (see, for example, Beinert & Stansly, 1953). If that were the case, such an exchange reaction might be expected to occur also in the presence of fumarate, so that the addition of carrier acetoacetate should alter the distribution of isotope in the metabolic products studied; e.g. the dilution of [^{14}C]acetyl-coenzyme A by exchange with inactive acetoacetate should result in a marked depression of the radioactivity of the respiratory carbon dioxide and of the fatty acids. However, when fumarate was present, the addition of carrier acetoacetate resulted in only a small decrease of the activity of the fatty acids (Table 4); on the other hand, the labelling of the acetoacetate was suppressed by the addition of fumarate in amounts large enough to stimulate lipogenesis (Tables 1 and 2). In the presence of fumarate in low concentration, the appearance of acetate carbon in acetoacetate was inhibited by *p*-nitrophenol to a greater extent than its oxidation to carbon dioxide and water (Tables 2 and 8). Further, a measurable net increase in the amount of acetoacetate was detected by means of a non-isotopic method of analysis (Table 2). These observations make it appear unlikely that in the present experiments an exchange reaction played a major part in the labelling of acetoacetate. The amount of substrate carbon appearing in the carrier may therefore be assumed to provide a measure of the capacity of the tissue preparation to form acetoacetate from the various precursors studied.

Factors influencing ketone-body formation and lipogenesis. Lack of oxaloacetate or its precursors is known to result in the formation of ketone bodies *in vitro*. Previous workers chose the experimental conditions so as to prevent or severely restrict the functioning of the Krebs cycle. Lehninger (1946) and Recknagel & Potter (1951) studied the conversion of pyruvate into acetoacetate by washed rat-liver suspensions poisoned with malonate. Crandall & Gurin (1949) employed a washed liver homogenate in a study of the formation of acetoacetate from labelled pyruvate, acetate and fatty acids. Catalytic amounts of Krebs-cycle intermediates have been found to be effective in preventing the accumulation of acetoacetate; e.g. Weinhouse, Millington & Volk (1950) reported that fumarate in concentrations as low as 0·1–0·5 mM suppressed the formation of acetoacetate from [^{14}C]palmitate. In the present work much higher concentrations of fumarate, in excess of amounts needed to catalyse Krebs-cycle oxidations, were required to abolish ketone-body formation and, at the same time, to stimulate fatty acid synthesis. The two processes were mutually exclusive only under extreme conditions, i.e. when the concentration of fumarate was either very high or very low. By choosing a suitable intermediate concentration of fumarate, it was possible to allow both acetoacetate formation and lipogenesis to proceed at reduced but measurable rates.

As concluded in the earlier paper (Terner, 1956*b*), the necessity to add fumarate in quantities greatly exceeding the small amounts required to catalyse Krebs-cycle oxidations suggested an additional role of fumarate as a stimulant of lipogenesis by the generation of reduced pyridine nucleotide.

That the availability of reduced pyridine nucleotide is a requirement for lipogenesis in non-respiring extracts has been demonstrated by various workers. According to Langdon (1955, 1957) and Brady, Mamoon & Stadtman (1956), reduced triphosphopyridine nucleotide (TPN) is required for the synthesis of fatty acids by extracts of liver, whereas Hele, Popják & Lauryssens (1957) found that rabbit mammary-gland extracts required reduced diphosphopyridine nucleotide (DPN). Brady *et al.* (1956), using extracts of acetone-dried powders of pigeon liver, added citrate and fumarate in high concentration (0·01–0·1 M) to regenerate reduced TPN. In work with respiring mammary homogenates, the addition of fumarate in increasing amounts (1–10 mM) has been found to result in a progressive stimulation of fatty acid synthesis (Terner, 1954, 1956*b*) and, as shown in this paper, also in a progressive decrease in acetoacetate formation.

Since lipogenesis is a reductive process, the accumulation of acetoacetate in mammary homogenates metabolizing acetate or pyruvate in the presence of catalytic amounts of the oxaloacetate precursor appears to be a consequence of the low reductive capacity of the tissue preparation. In the studies of lipogenesis quoted above, in which cell-free extracts and fractions were employed, reducing conditions were more easily maintained, owing to the diminution to a low level, or to the complete absence, of oxidative reactions. The present series of studies, with respiring mammary and kidney homogenates, represents an attempt to study the balance of oxidative and reductive reactions in a vigorously oxidizing system, which, although cell-free, may approach more closely than a non-respiring extract the conditions in the living organ.

Relative ketogenicity of various precursors of acetyl-coenzyme A. The marked contrast in ketogenicity between pyruvate and acetate on the one hand, and glucose on the other, observed in mammary and kidney homogenates is in agreement with previous findings (see Weinhouse, 1952) and emphasizes the long-felt need for an explanation of the role of carbohydrate in ketogenesis. There is no reason to assume that acetyl-coenzyme A derived from glucose is less capable of forming acetoacetyl-coenzyme A than if derived from other precursors. As pointed out by Krebs (1950), pyruvate is an obligatory intermediate in the breakdown of carbohydrate and it cannot be doubted that carbohydrate can serve as a source of ketone bodies; its antiketogenic action therefore remains to be explained.

The low ketogenicity of lactate, analogous to that of glucose, observed in the present work offers a possible explanation of the low ketogenic power of carbohydrate, at least in the present system. Since the conversion of lactate into acetyl-coenzyme A differs from that of pyruvate in only one additional oxidative step, coupled with the reduction of DPN, it seems that the ability, shared by glucose and lactate, to generate reduced pyridine nucleotide is the fundamental property which determines their low ketogenic capacity, contrasting with the powerful ketogenicity of acetate and pyruvate. As shown previously (Terner, 1955), citrate accumulates in mammary homogenates incubated with pyruvate or acetate in the presence of large amounts of fumarate. Under these conditions, the malic dehydrogenase reaction may yield reduced pyridine nucleotide. The availability of the latter would facilitate the reduction of acetoacetyl-coenzyme A, thus starting the chain of reactions leading to the formation of fatty acids (see Lynen & Ochoa, 1953). The reductive step would be in competition with the enzymic deacylation of acetoacetyl-coenzyme A and, in the absence of reactions maintaining an adequate level of reduced pyridine nucleotides, the prevalence of the deacylation reaction should result in the formation of acetoacetate.

Since acetoacetyl-coenzyme A was undoubtedly formed from pyruvate, it must also have been formed at comparable rates from lactate as well as from glucose, but in the 'non-ketogenic substrates' it could then have been largely reduced to β-hydroxybutyryl-coenzyme A. This is supported by the observation that the 'non-ketogenic' substrates yielded small but measurable amounts of acetoacetate (Tables 10 and 11). Since oxidative reactions are known to occur mainly within the mitochondria, whereas the reductive synthesis of fatty acids has been demonstrated to take place in the soluble extra-mitochondrial fractions of mammary tissue (Hele *et al.* 1957), spatial separation may provide an explanation. The glycolytic system is also located in the soluble fractions, and glucose may be more effective in making reduced pyridine nucleotides available to the soluble enzyme systems than Krebs-cycle oxidations.

The recovery of most of the added carrier acetoacetate (Table 2) indicates that its reduction by reduced DPN occurred at a relatively insignificant rate and that acetoacetate in its non-activated form played but a minor part in the reactions studied.

Effect of glucose on ketogenesis and lipogenesis. The mechanism outlined above may explain not only the low ketogenicity of glucose when added as the only oxidizable substrate, but also its stimulating effect on the synthesis of fatty acids from acetate, originally observed by Folley & French (1950) and Balmain *et al.* (1952) in rat- and sheep-mammary slices, and later also found to occur in cell-free guinea-pig mammary homogenates metabolizing [*carboxy*-^{14}C]acetate in the presence of high concentrations of fumarate (Terner, 1956*b*). The effect of glucose in these systems may be attributable to two factors: the enhanced rate of activation of acetate by the extra energy generated by the breakdown of glucose, and the maintenance of a high level of reduced pyridine nucleotide, in an oxidative environment otherwise unfavourable for reductive synthesis.

The apparent lack of antiketogenic power of glucose in the presence of acetate, shown in Table 7, may have been due to the experimental conditions being such that the rate of formation of acetoacetyl-coenzyme A from acetyl-coenzyme A, originating from the combined sources, exceeded the rate of generation of reduced pyridine nucleotide coupled with the breakdown of glucose alone, to an extent that a large part of the acetoacetyl-coenzyme A escaped reduction and suffered deacylation.

Ketogenesis in the mammary gland. The well-known fact that ruminants are especially susceptible to ketosis (see Shaw, 1956) may be connected with their ability to utilize acetate and fatty acids as a major source of energy. The demonstration of the utilization of acetate for the synthesis of fatty acids in slices of mammary gland from ruminants is due to the original work of Folley & French (1950) and Balmain *et al.* (1952), who also showed that the concurrent metabolism of glucose facilitated the utilization of acetate by non-ruminant mammary tissue. The guinea-pig mammary-tissue preparation used in the earlier paper (Terner, 1956*b*) and in the present work, resembles ruminant-mammary tissue in its ability to utilize acetate.

An organ such as the lactating mammary gland, subjected to conditions of considerable strain and

utilizing relatively large amounts of acetate, must depend on a large and continuous supply of reduced pyridine nucleotide in order to promote lipogenesis and suppress ketogenesis. If the supply of glucose were reduced, or its breakdown retarded, the balance precariously maintained might easily be shifted in favour of ketogenesis. It thus seems not improbable that at least part of the acetoacetate found in the milk of ketotic cows (Robertson & Thin, 1953) originates in the udder itself, instead of being derived entirely from the liver and rumen. To those two important sites of ketogenesis the udder may possibly therefore be added as a third.

SUMMARY

1. Conditions affecting the balance between ketogenesis and lipogenesis in actively respiring homogenates of the lactating mammary gland of the guinea pig have been studied.

2. [*carboxy*-^{14}C]Acetate and [2-^{14}C]pyruvate, when incubated with mammary homogenates in the absence of added fumarate, were oxidized to carbon dioxide and converted into acetoacetate at comparable rates. The addition of fumarate in increasing amounts (1–10 mM), which, as previously shown (Terner, 1956*b*), results in the progressive stimulation of fatty acid synthesis, caused a progressive inhibition of acetoacetate formation.

3. When unlabelled glucose was added to mammary homogenates metabolizing [*carboxy*-^{14}C]acetate in the absence of fumarate, it did not suppress and in some experiments promoted the incorporation of acetate carbon into acetoacetate. In the presence of both glucose and fumarate the formation of acetoacetate was abolished.

4. In marked contrast with the rapid rate of acetoacetate formation from acetate and from pyruvate, [^{14}C]glucose (randomly labelled) or [2-^{14}C]lactate gave rise to only small amounts of the ketone body. It is suggested that this difference resides in the ability of glucose and lactate to regenerate reduced pyridine nucleotide. The ability to maintain, in a vigorously oxidative system, conditions favourable for reductive reactions may be a common factor underlying not only the effects of fumarate on ketogenesis and lipogenesis in the present system, but also the well-known 'antiketogenic' properties of glucose and its stimulant action on lipogenesis.

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Separation and Composition of the Phospholipids of Ox Heart

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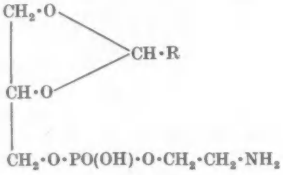
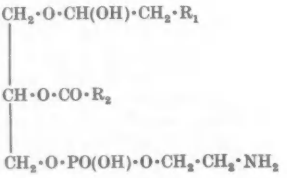
This work arose from a decision made in 1953 to prepare the acetal phospholipid described by Feulgen & Bersin (1939) and examine its susceptibility to various bacterial toxins. This acetalphosphatide, which at that time was regarded as a natural substance, is an acetal of a long-chain aliphatic aldehyde with glycerophosphorylethanolamine (I) from which aldehyde is liberated on treatment with acid or mercuric chloride. It was presumed that this compound, with only one fatty chain, could be readily separated from the classical ester phosphatide by chromatography. However, Schmidt, Ottenstein & Bessmann (1953) suggested from the results of hydrolysis of crude brain-lipid extracts that the natural 'plasmalogen' was a compound of

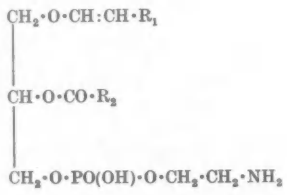
glycerophosphorylethanolamine acetal with other alkali-labile lipid groups. Shortly after the present work was begun Klenk & Debuch (1954) obtained substantial evidence that the natural ethanolamine plasmalogen of brain contained a fatty acid as well as a fatty aldehyde, and they suggested as probable the structure (II) or (III) in which the aldehyde was placed arbitrarily on the α-carbon atom of the glycerol. These authors predicted that, after



(I)



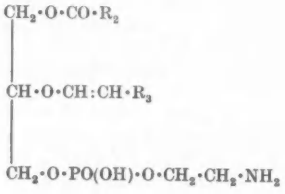
(II)



(III)

treatment of the natural compound with dilute acid to remove the aldehyde, lysokephalin would be obtained. This evidence was obtained by Debuch (1956), after the isolation by Klenk & Debuch (1955), on an analogous prediction, of lysolecithin as a product of the mild acid hydrolysis of the plasmalogen-containing lecithin fraction of ox-heart muscle.

The work of Klenk & Debuch (1954) provided sound evidence that the natural plasmalogens contain a fatty acid. The position was thus that the natural plasmalogens had never been isolated, that they existed as choline, ethanolamine and serine derivatives and that their intimate structure was unknown. Evidence of the structure (IV) of the ethanolamine and choline plasmalogens of ox heart (Gray, 1957*a*) and of the constituent aldehydes and fatty acids is given by Gray (1958*a*). While this work was in progress, Rapport, Lerner, Alonzo & Franzl (1957) and Debuch (1957) showed that the linkage of the aliphatic residue with glycerol, which is broken by mild acid treatment, giving the free aldehyde, has the αβ-unsaturated ether structure shown in (IV).



(IV)

The attempt described in this paper to prepare plasmalogens free from the classical ester phosphatides has been combined with a study of the nature and amounts of the phospholipids obtained from ox heart by two different methods of extraction and subsequently fractionated by chromatography on silicic acid. This method of fractionation, developed by Lea & Rhodes (1954*b*), had not been used with extracts containing acid-labile plasmalogens, and particular attention has been paid to the quantitative aspects and to the detection of minor components or of artifacts due to breakdown. Some data on the composition of cardiolipin and glycerophosphoinositide, and the use of a novel method for partition chromatography of phospholipids, are recorded.

Nomenclature. Feulgen & Bersin (1939), realizing that the acetal phospholipid (I) might be an artifact, suggested that the name 'plasmalogen' be retained for the natural lipid from which 'plasmal' (a mixture of aldehydes) was obtained by treatment with acid or mercuric chloride. In the intervening years this suggestion has been ignored and the names 'acetalphospholipid' and 'plasmalogen' used indifferently for natural and synthetic substances thought to be identical and now known to be different. Rapport & Alonzo (1955*b*) suggested the name 'phosphatidal choline' (or phosphatidal ethanolamine) for the plasmalogen compounds, but this word does not indicate the chemical structure, and its similarity to 'phosphatidylcholine' (lecithin) is confusing to the eye and ear. We have used 'plasmalogen' as a general name for the substances analogous to structure (IV); choline, ethanolamine and serine plasmalogen for the compounds characterized by the nitrogenous base; kephalin plasmalogen for fractions containing amino nitrogen; and lysoplasmalogen for compounds obtained by removal of the fatty acid from structure (IV). Removal of the aldehyde group from (IV) gives a lysolecithin or lysokephalin.

EXPERIMENTAL

Analytical methods

Aldehyde. The colorimetric method of Leupold & Buttner (1953) was used, with some adaptation. This is a two-stage reaction, the combined aldehyde being first split off in acid, and then condensed with the fuchsin reagent; this allows assessment of the acid-lability of plasmal compounds. Reagents: (i) 1·0 g. of rosaniline hydrochloride in 700 ml. of boiling water, filtered after cooling, 5·0 g. of $Na_2S_2O_5$ and 100 ml. of N-HCl, made up to 1 l., decolorized for 48 hr., stored at 2° in a dark glass-stoppered bottle with narrow neck. (ii) sulphite water (0·5% $Na_2S_2O_5$ in 0·1 N-HCl); (iii) Hopkin and Williams Ltd., 'capryl alcohol (octan-2-ol) "low in ketones" grade' was used for extraction of the coloured complex, as various brands of 'amyl alcohol' examined gave a high blank. For total aldehyde the lipid samples, together with two standards (10 and 20 μg. of palmitaldehyde as dimethylacetal) and a blank, were placed in glass-stoppered centrifuge tubes and the solvent was completely removed on a rotary evaporator. Acetic acid (0·5 ml. of 90%, v/v) was added, dissolving the lipid, and the tubes were kept at 50° for 45 min. Fuchsin reagent (2·0 ml.) was then added and after 20 min. at room temp. 2·0 ml. of sulphite water and 5·0 ml. of capryl alcohol were added. The tubes were shaken vigorously for 30 sec. and centrifuged for 4 min. The optical density of the coloured layer was measured in a Unicam spectrophotometer SP. 600 at 546 mμ or usually in an EEL colorimeter with filter 624. Free aldehyde, condensed in 90% acetic acid with ice-cold reagent at 2° for 30 min., gives 96% of the value obtained at room temp. but the dimethylacetal and native plasmalogen without previous hydrolysis give at 2° only 2–3% of the maximum.

The dimethylacetal of palmitaldehyde was used as standard. The method of Stephen (1925) for synthesis of palmitaldehyde failed (cf. Knight & Zook, 1953), but the method of Weygand, Eberhardt, Linden, Schafer & Eigen (1953), starting with palmitoyl chloride, gave palmitaldehyde in 85% yield. [The palmitoyl chloride was stated by the manufacturers, probably justifiably by classical criteria, to be 98% pure. Gas–liquid chromatography (see Gray, 1958*a*) showed that the aldehyde was a mixture of very closely related aldehydes, possibly including branched-chain forms. Apparently, 'pure' natural palmitic acid may be inhomogeneous.] The palmitaldehyde was refluxed with anhydrous methanol and benzene and a trace of toluene-*p*-sulphonic acid for 3 hr. and the water removed by azeotropic distillation. The dimethylacetal was isolated and redistilled twice *in vacuo*, giving an odourless, colourless liquid which solidified on storing at 2°. A stock standard was prepared by dissolving 48 mg. in 20·0 ml. of chloroform, and a working standard equivalent to 100 μg. of palmitaldehyde/ml. prepared by dilution.

The standard curve was linear in the range 5–50 μg. of aldehyde. On the basis of many readings with several batches of reagent, 10 μg. of palmitaldehyde in the test conditions gave a mean reading of 13·5$\pm$4% EEL divisions with filter 624, or an extinction of 0·17 in a 1 cm. cell at 546 mμ with reagent decolorized at least 48 hr. The readings became higher as the reagent aged with loss of SO_2; standards to which the test samples were referred were always included in the test, and the reagent was discarded when the normal colour value was significantly exceeded.

If the test sample contains much neutral fat, the mixture with the fuchsin reagent may be opalescent and a small amount of coloured solid may be left at the interface on extraction.

Comparison of the dimethylacetals of synthetic and natural aldehydes. As the colorimetric estimations were carried out on fractions containing a mixture of aldehydes and other phospholipids which might affect the result, the validity of the test was checked. A mixed lecithin–choline plasmalogen fraction contained by colorimetric assay 1·67 g. of aldehyde. The aldehydes were isolated quantitatively as their dimethylacetals (Klenk & Friedrichs, 1952); the yield was 1·85 g., equivalent to 1·62 g. of aldehyde by weight, and by colorimetric assay the product contained 1·59 g. of aldehyde. The colorimetric method therefore was a valid assay of the aldehyde content of the fractions. Since natural plasmalogens contain a mixture of aldehydes with differing colour values, results referred to a particular standard may not of course be absolutely correct.

Expression of results. As the molar ratio of P to aldehyde in plasmalogen is unity, the ratio moles of aldehyde/moles of P $\times$ 100 gives the P present as plasmalogen as a percentage of the total P in a fraction; this was called the plasmalogen value.

Amino nitrogen. The colorimetric method of Lea & Rhodes (1954*a*) was used, and the optical density measured in the EEL colorimeter with filter 626 with recrystallized ethanolamine hydrochloride as standard (0·7 and 1·4 μg. of N). The ninhydrin reagent was unstable in spite of precautions to exclude light and oxygen and was made up on the day required. The colour value for serine/μg. of N was 50% higher than for ethanolamine, but as in most of the fractions serine was present in very small proportion the method gave in general a reliable estimate of the ethanolamine nitrogen.

Fatty acid ester groups. The method of Rapport & Alonzo (1955*a*) was satisfactory, the optical density being compared in the EEL colorimeter, filter 624, with standards containing 1 and 2 μmoles of fatty acid ester. The colour yield with triolein and methyl palmitate increased slightly and that with lecithin decreased sharply if the time of heating at 65° with the alkaline hydroxylamine solution exceeded 60 sec. The colour yield also varies with the fatty acid present, or possibly with the complexity of the molecule; lecithin, lysolecithin, kephalin and lysokephalin gave 80–85% of the value with triolein or methyl palmitate per μmole of ester. A purified lecithin was therefore used as standard in analysis of phospholipid fractions, for which the method, though clearly not absolutely correct, is very useful.

Alkali isomerization of fatty acids. The method of Herb & Riemenschneider (1953) was adapted: 0·2 ml. (3–4 mg.) of fatty acids in methanol (0·2 ml. of methanol alone for the blank) was mixed rapidly with 3·0 ml. of 21% (w/w) KOH in redistilled ethylene glycol in glass-stoppered tubes which had been preheated 15 min. in a bath at 180°. The tubes were kept at 180° for 15 min. and then immediately cooled in water; the contents were transferred to a flask with methanol and made up to 25 ml. A further dilution (1:10) was read against the blank in a 1 cm. cell in the Uvispek spectrophotometer at 233, 268, 315 and 346 mμ. The results were calculated according to formula (*a*) of

Herb & Riemenschneider and ascribed to linoleic acid, linolenic acid, arachidonic acid and C_{20-22} polyenoic acids. The acids were not formally identified and authentic standards were not available; the results, however, agreed well with gas-chromatographic analyses carried out by Dr A. T. James and Miss J. Webb at the National Institute of Medical Research, Mill Hill, London, N.W. 7.

Phosphomonoester. Identification of compounds as phosphoric monoesters, $R{\cdot}PO_3H_2$, was made by finding the proportion of P hydrolysed at pH 8·6 in 30 min. at 38° by a bone phosphatase free from diesterase, added in amount sufficient to hydrolyse any monoester in these conditions.

Choline phosphatides. Phosphatidylcholine, choline plasmalogen and sphingomyelin are hydrolysed by *Clostridium welchii* lecithinase. The amount of P present as these compounds was ascertained by estimation of the amount of acid-soluble P formed in 30 min. at 38° and pH 7·4 in borate buffer containing 0·004M-$CaCl_2$ in presence of excess of *Cl. welchii* lecithinase.

Inositol. Duplicate samples of lipid were hydrolysed in sealed ampoules in 6N-HCl for 41 hr. at 115°, adjusted to pH 5·1 and diluted for microbiological assay by comparison with dose–response curves with known amounts of inositol. We are indebted to Dr T. B. Bright of The Distillers Co. Laboratories, Epsom, for a culture of *Saccharomyces carlsbergensis* and details of the culture medium. The results were reproducible within about ±10%.

Other methods. Estimations of phosphorus (Martland & Robison, 1926), total N (micro-Kjeldahl with Se catalyst), ethanolamine and serine (Axelrod, Reichenthal & Brodie, 1953), choline (Appleton, La Du, Levy, Steele & Brodie, 1953), glycerol (Blix, 1937), glycerolphosphate (Burmaster, 1946), iodine value (Trappe, 1938), cholesterol (Rose, Schattner & Exton, 1941) and methylglyoxal (Ariyama, 1928) were carried out by standard methods.

Solvents and general precautions. Chloroform (B.P.), methanol and anaesthetic ether were used as supplied for extraction, fractionation and most of the chromatography. For analytical work or solution of fractions ether was dried with Na wire, and ethanol was freed from esters by keeping over NaOH and redistilling. Lipid fractions were never kept solid; solutions were concentrated under reduced pressure in a stream of N_2, usually not above 35° bath temperature; the residual solvent was removed *in vacuo* at room temp. and the lipid immediately redissolved in the required solvent. Washed extracts or fractions were usually kept in chloroform containing 2% of methanol, and were dried with anhydrous Na_2SO_4 or $CaSO_4$ before storage under N_2 at −10° in glass-stoppered flasks, and in these conditions were remarkably stable.

Extraction and fractionation of phospholipids from heart muscle

Fractionation of phospholipids extracted with chloroform–methanol mixtures

The extraction was based on that of Folch, Ascoli, Lees, Meath & LeBaron (1951), but the amount of solvent was reduced for larger-scale work. Lean muscle from ox heart, obtained 2–3 hr. after death, was minced in a domestic mincer, and then blended in weighed portions with ice-cold solvent for 30 sec. in an M.S.E. blendor at room temp. and kept 15–20 min. in large jars with occasional stirring. The extract was filtered on Büchner funnels, and the residue was re-extracted. Four preparations were made by this method from 1·5 to 2·5 kg. of muscle and the following solvents: O_1, 2 vol. (l./kg.) of chloroform–methanol (2:1, v/v), re-extracted twice with 1 vol. of solvent; O_2, O_3 and O_4, 5 vol. of chloroform–methanol (1:1, v/v), re-extracted twice with 1 vol. of chloroform–methanol (2:1, v/v). Extracts O_1–O_3 were washed by stirring with 3–4 vol. of tap water; after 1–2 hr. the water was siphoned off, the voluminous white solid at the interface removed and drained, and the washing repeated, usually twice with tap water and once with distilled water at 2° overnight, till the volume of chloroform was constant. Extract O_4 was washed three times with 0·5% NaCl. The deep-yellow chloroform layer was put at −10° overnight to freeze the remaining water and filtered at 2°, and the solution dried over $CaSO_4$.

The yield and composition of the washed lipid, which included neutral fat and cholesterol, was substantially the same in the four preparations: 26–28 g. total wt., 570–600 mg. of P (about 14–15 g. of phospholipid)/kg. of fresh mince; molar ratio N:P between 0·9 and 1·1; amino N 37–42% of total N; plasmalogen value, 40–45%. Extract O_4 contained about 6% of the total N as serine, and 36% as ethanolamine; no inositol was detected. Extract O_1 contained 93–95% of the P as ester-P, estimated by the method of Schmidt, Benotti, Hershman & Thannhauser (1946), indicating that not more than 7% was present as sphingomyelin.

In a small-scale quantitative experiment, 10 g. of finely minced heart muscle was extracted with 20 vol. of chloroform–methanol (2:1, v/v) for 2 hr. at room temp. and washed as described by Folch *et al.* (1951); the chloroform solution contained 5·6 mg. of P (N:P ratio 1·1) and the interfacial solid contained 0·55 mg. of P (N:P ratio 3·9). The yield of phospholipid obtained in the large scale agrees with that in the small scale, and broadly with amounts found in heart muscle of various species in quantitative studies.

Separation of neutral fat from phospholipid. The phospholipid was separated from neutral fat by passing the solution through silicic acid with chloroform (Borgström, 1952). The phospholipids are quantitatively adsorbed, and subsequently eluted with methanol. The phospholipid was then rechromatographed on silicic acid (Lea & Rhodes, 1954*b*; Lea, Rhodes & Stoll, 1955) with chloroform with increasing concentrations of methanol, which displaces the phospholipids in the order phosphatidic acid, kephalin, lysokephalin, lecithin and lysolecithin.

Silicic acid prepared by the method of Isherwood (1946) was variable in properties. Silicic acid (Mallinkrodt A. R.) has been used throughout; the particle size is regular (100-mesh) and the flow rate was suitable without the use of a filter aid. The solvent used for elution was adjusted according to the properties of a particular batch of silicic acid within the range methanol–chloroform 1:9 to 1:4 for kephalin and 2:3 to 3:2 for lecithin. This silicic acid is, however, apparently so active that, in certain circumstances which are not fully under-

stood, breakdown of plasmalogens can occur on the column. This is shown in the following experiment in which identical portions of a lipid extract were run in succession through the same column, breakdown occurring in the first but not in the second run.

The washed lipid extract O_2 was divided into equal portions. The first portion, O_2a (385 mg. of P), in 250 ml. of chloroform was loaded on a column (diam. 40 mm.; 230 g. of silicic acid) and chloroform was passed under pressure (30–40 mm. Hg) of N_2 until the neutral fat was washed through; this required 1·75 l. and about 15 hr. The phospholipid, visible as a series of brown bands, was then eluted with chloroform–methanol (1:3, v/v) (2·5 l.; 30 hr.). The column was washed with chloroform till free from methanol and percolated overnight. The second portion of lipid extract O_2b was then chromatographed similarly. Analysis of the fractions from both runs showed an excellent recovery of P (97 %) in the phospholipid fractions, but 36 % of the plasmalogens in O_2a had been split, giving free aldehyde which appeared in the fat fraction, and presumably lysophosphatides. In the second run, O_2b, the plasmalogens originally present were recovered in the phospholipid fraction. Part of the cholesterol was retained with the phospholipid. A small amount of P ran in the fat fraction in the tail of the main cholesterol fraction.

The only difference in the two runs was that the silicic acid had been aged by the first run. It has been found in many subsequent runs that in the absence of the fat fraction the plasmalogens are stable on the silicic acid columns, and they are also stable for 14 days or so in chloroform solution containing the fat. It seems possible that there can be a reaction between very active silicic acid and some component of the fat which increases the acidity of the column sufficiently to decompose the acid-labile plasmalogens.

Fractionation of phospholipids on silicic acid. (i) A portion of phospholipid fraction O_2a (145 mg. of P) which presumably contained lysophosphatide derived from plasmalogens, was chromatographed on a column (diam. 31 mm., height 280 mm.) containing 100 g. of silicic acid in methanol–chloroform (1:4, v/v). The kephalin band was eluted with this solvent and elution continued with methanol–chloroform (1:1) until the lecithin band was off. The separation curve based on analyses of 15 ml. fractions for P, amino N and aldehyde is shown in Fig. 1. A second portion of O_2a chromatographed in the same way gave an almost identical curve. The fractions from both runs were pooled on the basis of the curves into four fractions (approximately phosphatidic acids, kephalin, lysokephalin and choline phosphatide) *A*, *B*, *C* and *D*, the analyses of which are given in Table 1.

(ii) A portion of phospholipid fraction O_2b (66 mg. of P), in which the plasmalogens were intact, was chromatographed by the same procedure on 40 g. of silicic acid. The separation curve (8 ml. fractions) is shown in Fig. 2. The fractions were pooled into four main fractions *A*, *B*, *C* and *D* as before (Table 1).

In both runs, the front band *A*, containing 12–15 % of the total P and a little cholesterol, was dark brown and low in N, and was presumed to be mainly cardiolipin (see p. 420). The kephalin fraction *B*, brownish yellow, contained 24 % of the

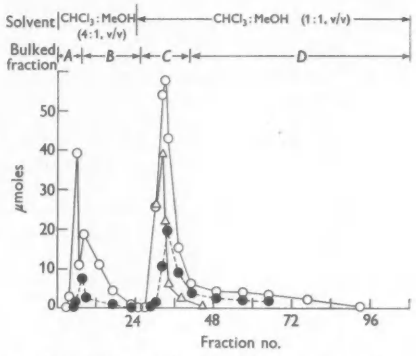


Fig. 1. Fractionation of phospholipid extract O_2a on silicic acid. ○, Total P; ●, plasmalogen P, calc. from aldehyde content; △, amino N.

Table 1. *Separation of components of the phospholipid preparations* O_2a *and* O_2b

O_2a: 290 mg. of P; recovered from column, 278 mg. O_2b: 66 mg. of P; recovered from column, 62 mg.

Component	Fractions from O_2a				Fractions from O_2b			
	A	*B*	*C*	*D*	*A*	*B*	*C*	*D*
P (mg.)	33	67	78	100	9	22	11	20
N (mg.)	4	29	39	48	0·5	10	5	10
Amino N (mg.)	2	28	23	2	0·6	11	1	0
Aldehyde (mg.)	27	80	131	445	5	82	32	109
P (% of recovered P)	12	24	28	36	14	36	18	32
% of P in fraction as kephalin	13	90	60	4	14	100	20	Nil
% of P in fraction as plasmalogen	10	15	20	55	6	45	36	67

total P in O_2a, against 36 % in O_2b, and the plasmalogen value was 15, against 45 %, a decrease equivalent to 12 % of the total P. The lysokephalin content of fraction *C* was calculated from the ratio amino N/total N; from this, the percentage of the total P present as lysokephalin was estimated as 16 % in O_2a and 4 % in O_2b. The loss of kephalin plasmalogen in O_2a was therefore almost exactly balanced by the appearance of lysokephalin. The colourless fraction *D* contained no amino N; the total amount of choline phosphatides, a mixture of lecithin and choline plasmalogen, calculated as the sum of *C* and *D*, minus lysokephalin, was equivalent to 48 % of the total P in O_2a and 46 % in O_2b, with plasmalogen values of 56 and 61 % respectively, showing that there had been little, if any, breakdown of choline plasmalogen. About 95 % of the P was recovered. The sphingomyelin band was not eluted.

If the amino N in fraction *C* is regarded as lysokephalin derived from the breakdown of ethanolamine plasmalogen, the distribution of the lipid P in the chloroform–methanol extract of heart was approximately cardiolipin 12, kephalin (ester phosphatides) 22; ethanolamine plasmalogen, 20; lecithin, 21; choline plasmalogen 25 %.

Fractionation of phospholipids extracted with methanol followed by chloroform

Several extractions (O_5–O_{10}) were made by the method of Pangborn (1945), i.e. extraction with methanol after dehydration and defatting with acetone, and recovery of the acidic phospholipids as the barium salts. About 80 mg. of lipid P/kg. of mince was lost in the acetone extracts. The amount of lipid P extracted with methanol was less than with chloroform–methanol; re-extraction of the residue with chloroform yielded a further amount of phospholipid. The protocol of one experiment follows.

Protocol. O_{10}. Ox heart (7·25 kg.) was blended mechanically with the solvent and extracted twice with 1·2 l. of acetone/kg. The residue was extracted with 2 l. of methanol/kg. of mince in large bottles at 2° for 4 days, with occasional shaking, then filtered off and washed on the funnels with methanol. (The extraction of phospholipid was 94 % of the maximum in 24 hr.) The yellow extract (17 l.) was treated with aqueous 40% $BaCl_2$, and the clear supernatant collected by decantation and centrifuging. The precipitated Ba salts, containing acidic phospholipids, nucleotides and inorganic phosphate, were quickly washed in 20 % (v/v) acetic acid, and the ether-soluble Ba salts separated into two fractions by dissolving three times in ether and reprecipitating with 2 vols. of methanol. The methanol-insoluble Ba salts were converted into Na salts (fraction *A*, Table 2; total P, 350 mg.); the methanol-soluble supernatant (fraction *B*) contained 120 mg. of P.

The main methanol extract, after removal of the insoluble Ba salts, was concentrated to 3 l.; an equal volume of chloroform was added, and the mixture washed three times with about an equal volume of water. The washed chloroform solution was concentrated and dried (fraction *C*, 2430 mg. of P).

The mince residue, after the extraction with methanol, was stirred with chloroform (about 700 ml./kg. of mince) and filtered off after a few hours at room temperature. The extract was highly pigmented and contained neutral fat. An equal volume of methanol was added, and the mixture washed with water. The washed chloroform solution was concentrated to about 400 ml. and poured into 4 vols. of acetone. The precipitated phospholipid was dissolved in chloroform, giving a brown solution (fraction *D*, 630 mg. of P); the acetone supernatant contained 42 g. of solids, but only 30 mg. of P.

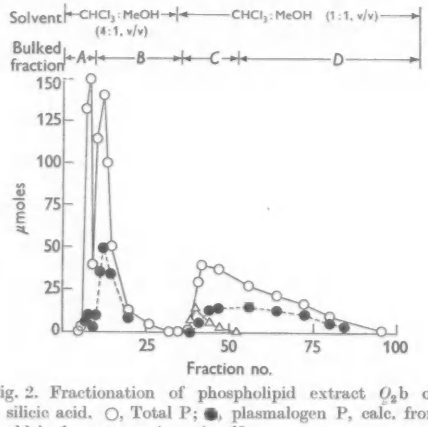


Fig. 2. Fractionation of phospholipid extract O_2b on silicic acid. ○, Total P; ●, plasmalogen P, calc. from aldehyde content; △, amino N.

Chromatographic fractionation of fractions C *and* D

Portions of fractions *C* and *D*, each containing about 120 mg. of P and a little fat, were chromatographed on silicic acid in identical columns by the usual procedure. The separation curves are shown in Fig. 3*a* and *b*. Fraction *C* separated into three distinct bands corresponding approximately to kephalin, lecithin and sphingomyelin. Fraction *D* gave three similar bands and two small fast-running bands. The eluate fractions were pooled as shown in Table 2, the front and rear portions of each of the kephalin and lecithin bands being kept separate. Each pool was concentrated and analysed without further purification. The distribution and analyses are shown in Table 2.

The total lipid P recovered in the fractions *A*–*D* was 490 mg./kg. of mince, which, with the additional amount lost in the acetone, is in good agreement with the chloroform–methanol extracts (p. 412). The methanol extract contained 82 % of the total P, but there was some differential extraction, as this extract contained about 95 % of the choline phosphatides, but only 70 % of the

Table 2. *Distribution and analysis of phospholipids in fractions from methanol and chloroform extracts of ox heart*

Fraction		P content as % of total lipid P	Analysis of fraction: % P on dry wt.	Molar ratio (P = 100): Total N	Amino N	Aldehyde	Fatty acid ester	Double bonds/mol. of fatty acid
Methanol extract								
A		10·0	3·5	16	20	6	190	—
B		3·0	—	—	97	30	—	—
C		69·0	—	—	38	56	—	—
Subfractions of *C*								
Tubes	Fraction							
35–41	3	5·0	3·7	99	90	33	167	1·99
42–61	4	18·4	4·1	96	100	58	152	1·91
71–84	5	4·7	3·6	106	16	37	162	2·02
85–120	6	36·6	4·0	99	Nil	59	148	1·21
121–165	7	4·2	3·3	214	Nil	Nil	Nil	—
Chloroform extract								
D		18·0	—	—	66	45	—	—
Subfractions of *D*								
Tubes	Fraction							
10–31	1	2·2	4·2	4	12	17	190	1·64
32–38	2	1·6	3·5	—	25	6	185	1·93
39–51	3	8·5	3·8	91	100	43	165	1·86
52–85	4	3·2	3·8	87	100	46	150	1·43
86–96	5	0·1	—	—	66	14	—	—
97–124	6	1·8	3·8	98	Nil	51	148	1·13
125–176	7	0·5	2·7	101	Nil	Nil	100	—

kephalin phosphatide. The analyses (Table 2) indicate that the extraction into methanol is influenced by the nature of the fatty acids; calculated from the iodine uptake (after correction for the iodine uptake of the $\alpha\beta$-unsaturated linkage of the aldehyde component) the kephalin and choline phosphatide fractions in the methanol extract had a higher degree of unsaturation than those in the chloroform extract.

Nature of the phospholipids in the various fractions (Table 2)

Acidic phospholipids. Fraction *A*, containing the acidic phospholipids with Ba salts insoluble in methanol, is a mixture containing about 20% of inositide and 16% of nitrogen-containing phosphatide, including almost all the detected serine phosphatide (serine N, 60% of total N in fraction *A*). The remainder of this fraction is presumably cardiolipin, but a satisfactory quantitative separation of the components present has not yet been achieved (see p. 421).

The chloroform extract *D* on chromatographic fractionation gave two small fast-moving bands (D_1 and D_2), low in amino nitrogen. Fraction D_2 was in the usual position for the cardiolipin band, but it was thought that the fraction D_1, eluted in chloroform alone, might be a simple phosphatidic acid, either a natural intermediate or an artifact formed by breakdown of lecithin or kephalin. The constituent fatty acids were therefore isolated from a portion of D_1 after alkaline saponification, as a pale-yellow oil, iodine no. 161; no solid acids separated at 2°; alkali isomerization indicated that 82% of the total acids were present as linoleic and linolenic acids. The water-soluble P ester obtained after saponification in methanolic 0·2 N-NaOH for 15 min. at 37° gave only 45% of the P as glycerol phosphate after treatment with periodate at 100° in N-H_2SO_4 (cf. Table 7). No Na or K was detected in fractions D_1 and D_2. It was concluded that D_1 was substantially cardiolipin, possibly in a 'polymerized' form (see p. 421).

Ester phosphatides. Fractions C_3, C_4, D_3 and D_4 (Table 2) are substantially mixtures of phosphatidylethanolamine and ethanolamine plasmalogen; the serine N was less than 10% of the total N in these fractions. The slightly low N content of D_3 and D_4 may indicate the presence of a nitrogen-free phosphatide in amounts equivalent to about 1% of the total lipid P. Fractions *C* and *D* are mixtures of phosphatidylcholine and choline

plasmalogen; all the P in these fractions was hydrolysable by *Cl. welchii* lecithinase.

Lysophosphatides. Lysokephalin moves with the front of the lecithin band. Fractions C_5 and D_5 contained amino N equivalent to about 0·8 % of the total lipid extracted as lysokephalin. No evidence of the presence of lysolecithin was found, though it is impossible to exclude it absolutely.

Sphingomyelin. Fractions C_7 and D_7, obtained from small peaks in the tail of the lecithin band, were substantially sphingomyelin; the ester P was only 11–14 % of the total P. The low percentage of P on the dry wt., due to traces of silicic acid in the relatively large volume of solvent required for elution, was raised to 3·9 % after precipitation with ether from chloroform solution.

Composition of ox-heart phospholipids

From the analyses in Table 2 the distribution of phospholipids was computed (as % of the total lipid P) as: inositide, 2; cardiolipin (or similar compounds), 9; serine derivatives, about 5; phosphatidylethanolamine, about 20; ethanolamine plasmalogen, 18; phosphatidylcholine, 18; choline plasmalogen, 24; sphingomyelin, 5 %. The amounts are very similar to those obtained by fractionation of the chloroform–methanol extract (p. 414).

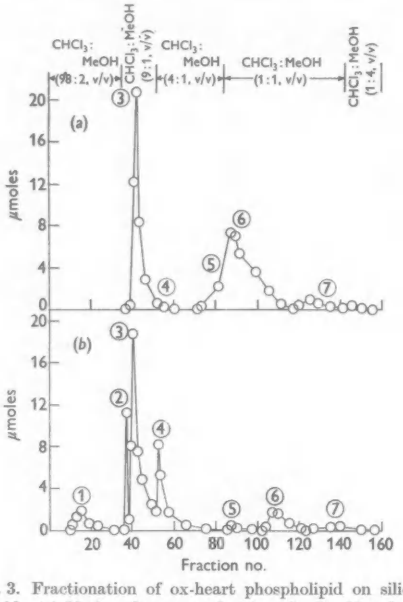


Fig. 3. Fractionation of ox-heart phospholipid on silicic acid. (*a*) Methanol extract, fraction *C*; (*b*) chloroform extract, fraction *D*. ○, Total P. The ringed numbers indicate the subfractions (Table 2).

The foregoing experiments show that fat-free phospholipid extracts containing plasmalogens can be chromatographed on silicic acid without breakdown and with good separation of the main kephalin- and choline-based components from each other. But, although the rear portions both of the kephalin and lecithin bands were richer in plasmalogens than were the front portions (see Fig. 1), the degree of separation was too small to be useful for the preparation of pure plasmalogens. A further attempt to separate plasmalogen from ester phosphatide on silicic acid was made by means of the gradient-elution technique developed by Lakshmanan & Leiberman (1954), but the enrichment was of no practical value. The non-polar groups of the phospholipids have clearly too small an influence on the degree of adsorption (cf. Lea *et al.* 1955) to permit separation of the ester and plasmalogen families from each other.

Fractionation of phospholipids by partition chromatography

The chances of separating a plasmalogen from the analogous ester phosphatide should be increased if the influence of the polar part of the molecule is decreased and the difference in the non-polar part is accentuated. Gray (1957*b*) pointed out that partition chromatography with purely organic solvents should be particularly useful for the separation of lipids and found that a secondary cellulose acetate, acetyl value 52·5 %, was suitable as a support.

Preliminary experiments were carried out with a lecithin–choline plasmalogen fraction and several solvent systems to see (*a*) whether there was a significant difference in the partition function of choline plasmalogen and lecithin, (*b*) whether the stationary phase was a good solvent for phospholipids, and (*c*) whether the partition functions of these phospholipids varied with their concentration.

Quantities of the two solvents of each system were shaken together and the equilibrated phases were separated and used as the appropriate stationary or mobile phase. The phospholipid (about 1·0 mg. of P) was dissolved in 2·0 ml. of the equilibrated stationary phase and shaken for a set time with 2 ml. of the mobile phase. After separation of the layers the mobile phase was removed and analysed for P and aldehyde content. Sufficient of the stationary-phase solvent was added to keep the phase volume constant, and the procedure was repeated 5–10 times. The percentage of choline plasmalogen in both phases was calculated. The results were disappointing in that any difference in the partition of the two components was within the experimental error, i.e. less than 8 % with the solvents tested.

The partition functions of the two compounds were so similar that it was possible to treat the phospholipid mixture as a single solute dispersed between the stationary- and mobile-phase solvents. The concentrations of the mixture in both phases of several solvent systems were plotted (Fig. 4*a*). The logarithmic values of the concentrations were also plotted and were found to be on a straight line (Fig. 4*b*). Therefore, the forms of the curves (distribution isotherm) were those covered by the Freundlich isotherm equation:

$$C_s = KC_m^n,$$

where C_s is the concentration of solute in the stationary phase, C_m the concentration of solute in the mobile phase, and K and n are constants. This showed that the partition function of the phospholipid solute varied with the concentration.

As even a 5% difference in partition function between the plasmalogen and the ester phosphatide might be enough for separation with solvent systems in which the R of both solutes is low, a number of partition columns were examined. A full account of these is given by Gray (1958*b*). The secondary cellulose acetate used was a standard commercial product of British Celanese Ltd., grade CRIFS, acetyl value 52·5–53·5%, supplied as a powder (50-mesh). The phospholipids chromatographed were mixtures of lecithin and choline plasmalogen, similar to fraction C_6, Table 2.

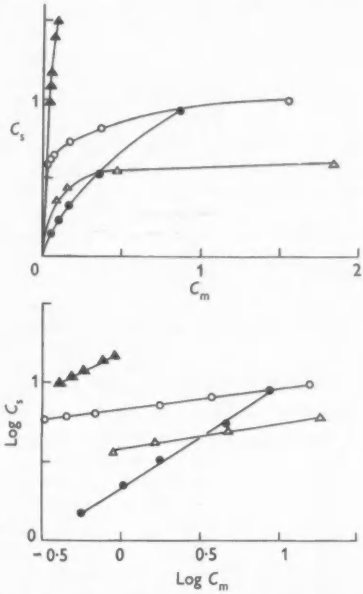


Fig. 4. Distribution of choline phosphatide between two immiscible organic phases. C_s = concentration of solute in stationary phase. C_m = concentration of solute in mobile phase. Phases (mobile first): ○, triethylene glycol–carbon tetrachloride; ●, diethylene glycol–ethyl butyrate; △, propylene glycol–carbon tetrachloride; ▲, carbitol–hexane.

(i) *Solvent system propylene glycol–carbon tetrachloride*

This system gave promising results but was abandoned because some breakdown of plasmalogen occurred. This was traced to the presence in the propylene glycol of some impurity, possibly secondary halides, giving rise to HCl. Even after refluxing with 70% NaOH, fractional distillation and shaking with Bio-Deminrolit (The Permutit Co. Ltd., London, W. 4), the propylene glycol available when mixed with carbon tetrachloride gave free acid within 48 hr.

(ii) *Solvent system light petroleum–methanol*

Column 42. The equilibrated stationary (methanol) phase (100 ml.) was mixed with 80 g. of cellulose acetate and enough mobile phase was added to give a thin slurry, which was shaken for 1 hr. The column (20 mm. diam.) was built up to a height of 600 mm., a head of light petroleum (b.p. 60–80°) being kept above the stationary phase to prevent bubble formation. It was necessary to lag the column and solvent container, as a change in temperature disturbed the equilibrium; and several lamps were connected with a thermostat at 22 ± 0·5° round the apparatus. The column was tested for uniformity of packing by running through a mixture of Sudan III and Sudan IV dyes.

The phospholipid (1·25 mg. of P; plasmalogen value 54%) was chromatographed at a flow rate of 5·0 ml./hr. under positive nitrogen pressure of 50 mm. Hg, and fractions were collected every hour. Approximately three column volumes (190 ml.) of eluate were collected before phospholipids appeared in the fractions. The first two fractions, containing 4% of the total P, contained only 20% of plasmalogen. In the next few fractions the plasmalogen value rose, to as high as 90% in two tubes, and then gradually decreased to only 11%. The eluate fractions were pooled into five main fractions, *A*–*E*, which were analysed (Table 3). Fraction *B*, which contained 72% of choline plasmalogen, an increase of 18% on the original material, was rechromatographed on a small column (no. 43), diam. 11 mm., height 350 mm., flow rate 2·0 ml./hr.; 27% of the P was recovered in a fraction with a plasmalogen value of 86%. The overall yield was 9% of the total P in the starting material, which had a plasmalogen value of 54%, i.e. 14% of the plasmalogen P was recovered as 86% pure.

It is apparent that the R values of some of the lecithins are almost the same as those of the plasmalogens in this solvent system. The spread of the band in column 42 was greater than would be expected for the amount of separation actually obtained, but as the two compounds are presumably both present as families the spreading of the band could be due to fractionation of members of each family according to the constituent fatty acids. The tailing was probably caused by the fact that the partition function of a phospholipid solute between a two-phase solvent system (Fig. 4) varies with concentration; as the concentration of the phospholipid in the more polar solvent became less the ratio of the phospholipid in the polar phase to that in the non-polar phase became greater. Therefore as the concentration of phospholipids decreased more of the mobile phase was required to elute them, so that the tail, though containing very little of the total phospholipid, was extremely long.

(iii) *Solvent system* cyclo*hexane–methanol*

In the light petroleum–methanol solvent system the R of the front fractions was approximately 0·33. The partitioning of the phospholipids was more in favour of the methanol in a *cyclo*hexane–methanol system than in the light petroleum–methanol system, from which it was inferred that on a partition column the R values would be lower.

Column 45. A column (diameter 10 mm., height 380 mm.), containing 14·5 g. of cellulose acetate supporting the methanol stationary phase, was equilibrated with the *cyclo*hexane mobile phase at 22°. Good lagging and temperature control were necessary. A lecithin–choline plasmalogen fraction (plasmalogen value 57 %; 0·5 ml.; 1·24 mg. of P) was settled in and elution was begun with a flow rate from the column of 3·0 ml./hr. under a positive nitrogen pressure of 150 mm. Hg. Fractions were collected every hour and analysed for P and aldehyde, and the separation curves for the choline plasmalogen and the lecithin plotted (Fig. 5). The R of the first phospholipid fractions was approximately 0·22. A good enrichment in choline plasmalogen was obtained in fractions 21–28, some fractions having a plasmalogen value of more than 90 %. Fig. 5 shows a small band of phospholipid (fractions 15–19) with a low plasmalogen value running ahead of the main choline-plasmalogen fractions; no amino nitrogen was found in this band. It was thought most probable that the band contained a lecithin which, because of the nature of its fatty acids, was slightly less soluble in methanol than was the main lecithin fraction and which was therefore eluted a little in front of the other lecithins.

The eluate fractions were pooled to give seven main fractions. The results (Table 4) show a small improvement over those obtained with the light-petroleum–methanol system on column 42, which was a much longer column. A bigger column was used for fractionation of larger quantities.

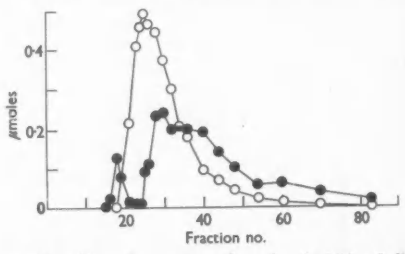


Fig. 5. Partition chromatography of a lecithin–choline plasmalogen mixture on cellulose acetate. Column 45. Solvent system: *cyclo*hexane–methanol. ○, Choline plasmalogen P, calculated from the aldehyde content; ●, lecithin P, calculated as total P minus plasmalogen P.

Table 3. *Partition chromatography of a lecithin–choline plasmalogen mixture*

Column 42. Stationary phase, methanol. Mobile phase, light petroleum (b.p. 60–80°).

	Original material	Fractions				
		A	*B*	*C*	*D*	*E*
Plasmalogen value (%)	54	20	72	61	51	11
P in fraction (% of total)	100	4	34	39	4	19

Table 4. *Partition chromatography of a lecithin–choline plasmalogen mixture*

Column 45. Stationary phase, methanol. Mobile phase, *cyclo*hexane. The mixture contained 57 % of choline plasmalogen.

	Fractions						
	I	II	III	IV	V	VI	VII
Tubes	15–19	21–28	29–41	42–51	52–63	64–76	77–90
P as % of total P	2	28·5	33·8	16·9	7·6	7·6	3·6
% of P in fraction as plasmalogen	10	85	62·5	39	28	13·5	12·4

Table 5. *Iodine values of successive fractions of a lecithin–choline plasmalogen mixture obtained on a cellulose acetate partition column with* cyclo*hexane–methanol*

	Fractions					
	A	*B*	*C*	*D*	*E*	*F*
P (μmoles)	72	216	110	88	52	13
Plasmalogen (μmoles)	58	127	59	41	23	5
Fatty acids (μmoles) calc.*	86	305	161	135	81	21
Iodine uptake (μmoles)	226	577	284	219	125	29
Iodine uptake of fatty acids (μmoles)†	168	450	225	178	102	24
Double bonds/fatty acid	1·95	1·47	1·40	1·32	1·26	1·13

* Calc. on 2 moles of fatty acid/mole of P for ester phosphatide, 1 mole/mole of P for plasmalogen. Ester phosphatide = μmoles of P − μmoles of plasmalogen.

† Corrected for I_2 uptake of aldehyde, 1 μmole/μmole of plasmalogen.

Column 53. A column (diameter 39 mm., height 670 mm.), containing 238 g. of cellulose acetate supporting the methanol stationary phase, was equilibrated with the mobile phase. It was much more difficult to obtain a uniform and tight packing with the large column and slight distortion of the band fronts and more band-spreading occurred. Lecithin–choline plasmalogen (20 mg. of P; plasmalogen value 57 %) was eluted at a flow rate of 20 ml./hr. under a positive nitrogen pressure of 50 mm. Hg. Fractions were collected every hour and analysed for P and aldehyde. There was a slightly lower degree of resolution of the lecithin and choline plasmalogen on the larger column; with the small column 45 the front fractions (fraction II, tubes 21–28) contained 28·5 % of the total phospholipids with a plasmalogen value of 85 %; with the large column 53 the corresponding fractions contained 20 % of the total phospholipids with a plasmalogen value of 79 %.

Effect of the constituent fatty acids on the fractionation. It was suggested above (p. 418) that the asymmetric shapes of the separation curves obtained (Fig. 5) were due to some separation of the individual lecithins and plasmalogens, owing to differences in the chain length and unsaturation of the constituent fatty acids.

The iodine values were estimated on the fractions from another fractionation of a lecithin–choline plasmalogen mixture with the partition system *cyclo*hexane–methanol. The results (Table 5) showed that the front fractions contained more highly unsaturated acids than did the back fractions, indicating that the fatty acids in the plasmalogens were more unsaturated than those in the lecithins. This fact was confirmed later by examination of the fatty acids of pure lecithin and pure α-acyl- and β-acyl-lysolecithins prepared from a lecithin–choline plasmalogen mixture (Gray, 1958*a*).

A number of fractionations of phospholipid mixtures with a choline plasmalogen value of between 50 and 60 % have been carried out on a preparative scale with the large column. In general the method yielded approximately 20 % of the total phospholipids as fractions with plasmalogen values of 75–85 %. Mixtures of kephalin and ethanolamine plasmalogen were also fractionated on cellulose acetate columns with a *cyclo*hexane–methanol solvent system. The results obtained were similar to those with the choline-containing mixtures, the front phospholipid fractions being enriched in plasmalogens whereas the rear fractions were richer in kephalins. The disadvantage of the *cyclo*hexane–methanol system for the separation of kephalin mixtures was the low solubility of the phospholipid in methanol, which caused it to pass through the column much too quickly for a reasonable resolution to be accomplished.

Excellent separations were obtained when lecithin–lysolecithin mixtures were chromatographed on cellulose acetate with a *cyclo*hexane–methanol system. The lecithins were eluted first, well separated from the lysolecithins, which were eluted very slowly in *cyclo*hexane but could be rapidly recovered by passing methanol through the column.

Many variations of solvent systems are possible with these partition columns and some may give better separations than those so far tried. However, the *cyclo*hexane–methanol system with cellulose acetate as support has been satisfactory for the preparation of plasmalogen-rich samples of phospholipid. The conditions on the columns are so mild that even a slight breakdown of a phospholipid is unlikely to occur, in contrast with systems in which the adsorbent is acid (silicic acid) or alkaline (alumina). The phospholipids can be quantitatively recovered, the column can be standardized and, provided that it is equilibrated after each fractionation, used repeatedly.

Cardiolipin

Cardiolipin was isolated from ox heart by Pangborn (1942) and is used as a constituent of the Wasserman antigen in the diagnosis of syphilis;

a method for its preparation within certain limits of purity for this purpose has been prescribed (Pangborn, Almeida, Maltaner, Silverstein & Thompson, 1955), involving repeated precipitations of the barium, sodium and cadmium salts.

Cardiolipin was considered by Pangborn (1947) to be a glycerylglycerophosphate derivative containing three phosphoric acid molecules linking four glycerol molecules, the latter being esterified with six fatty acids, in the ratio five linoleic to one oleic residue. This constitution was suggested on the basis of the best fit of the analytical data for the original compound, and for the polyglycerophosphate obtained by mild saponification, and it has never been proved. A bar to further work is the difficulty of purification by the standard method described for the preparation of cardiolipin as a serological reagent, and attempts were therefore made to prepare cardiolipin chromatographically.

Preparation of cardiolipin

(*a*) *Chromatography on silicic acid.* (i) The front fractions, mainly kephalin, obtained by chromatography on silicic acid of a phospholipid extract (O_3, p. 413) were re-run on a silicic acid column, beginning the elution with methanol–chloroform (1:9, v/v). Fig. 6 shows the separation of the presumed cardiolipin band (fraction *A*) from kephalin (fraction *B*). Fraction *A* was very dark-coloured, and was precipitated three times from concentrated solution in ether with acetone, which removed most of the pigment (Found: P, 4·0; N, 0·12; aldehyde, 0·6; glycerol, 17·8%; $[\alpha]_{5461}^{22}$ +8° in $CHCl_3$) (*c*, 3·7); molar ratio P:glycerol:fatty acid ester, 1:1·5:2. By gas-chromatographic analysis of the methyl esters the fatty acids contained about 80% of linoleic + linolenic acids. This material contained no Na or K, and after storing some time became insoluble in ether; it was then serologically inactive, presumably due to some change in the free acid with time (cf. Pangborn, 1944). It was concluded that the material was cardiolipin, the only obvious discrepancy being a molar ratio of glycerol to P of 3:2, instead of 4:3 on Pangborn's structure.

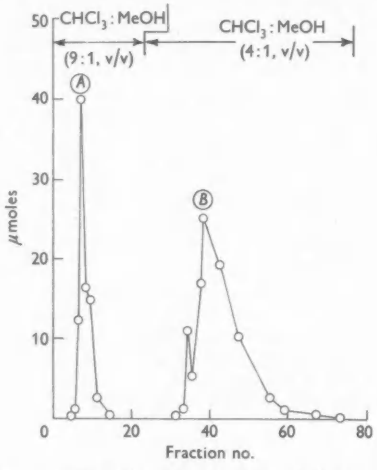


Fig. 6. Separation of cardiolipin and kephalin on silicic acid. Total P. Molar ratio P:N, fraction *A*, 1:0·03; fraction *B*, 1 : 0·94.

(ii) A methanol extract of heart muscle (O_5), prepared by Pangborn's (1945) method (p. 414), was concentrated *in vacuo* and the phospholipid extracted into chloroform. After being washed with water and dried, the chloroform solution (P, 9; Na, 5·7; K, 2·8 m-moles) was chromatographed on silicic acid, elution being begun with methanol–chloroform (1:19, v/v). The dark-coloured front fraction (about 5% of the total P) was concentrated, and the phospholipid was precipitated from ether with acetone, removing most of the pigment, with some loss of P, and dissolved in ether. The solution contained P 258, amino-N 4, Na 4·3, K 0 μmoles. The Ba salt was immediately prepared by pouring the ethereal solution into 2 vol. of methanol containing 0·2 ml. of 20% aqueous $BaCl_2$, and was reprecipitated from ethereal solution with methanol. It was converted into the Na salt by shaking the ethereal solution with 0·1 vol. of ethanol and 5% aqueous Na_2SO_4; the ethereal solution was dried and evaporated and the residue dissolved in dry ethanol. After 48 hr. at 2° the solution (O_5/b) was decanted. This material was serologically active.

(*b*) *Combination of fractional precipitation and chromatography.* The acidic phospholipids were precipitated as Ba salts from a methanol extract of heart muscle (O_7, p. 414). The Ba salts were purified by washing with acetic acid, and reprecipitating several times from ethereal solution with methanol, and were converted into Na salts. The material (molar ratio P:amino N:inositol, 1:0·2:0·2) was dissolved in chloroform and chromatographed on silicic acid, beginning with methanol–chloroform (1:19, v/v). The front, fast-running band contained 8% of the total P and no Na or K. The middle fractions, O_7/16, contained 10% of kephalin and 10% of inositide and the rear fractions, O_7/20, about 50% of inositide. The fraction O_7/16 was rechromatographed on silicic acid; 50% of the P was recovered in the front fraction, free from inositide, but containing 10% of kephalin. This fraction was further purified by dissolving the salt in ethanol (about 1%) and decanting the solution (O_7/16S) after 48 hr. at 2°.

(*c*) *Method of Faure & Morelec-Coulon* (1956). The acidic phospholipids in a methanol extract (O_9) of heart muscle were separated and purified as the Ba salts. The free acids were obtained in ethereal solution by treatment with methanolic HCl and saline, and fractionated in concentrated ethereal solution by addition of ethanol; cardiolipin remained in solution, practically free from nitrogen and inositide. It was recovered as the Ba salt and converted into the Na salt. The ethanolic solution (O_9/9S) of the Na salt was decanted after 48 hr. at 2°. The yield was about 4% of the lipid P.

Table 6 shows the analyses of these preparations. The serological activity of O_5b and O_9/9S was tested by Dr A. E. Wilkinson and compared satisfactorily with that of a standard cardiolipin preparation, but extensive tests were not made. The calculated figures in Table 6 for alternative

Table 6. *Composition of cardiolipin preparations*

(*a*) Pangborn (1945); (*b*) Faure & Morelec-Coulon (1956). (*c*) $C_{120}H_{205}O_{24}P_2Na_3$: 5 linoleic, 1 oleic residue (Pangborn, 1947). (*d*) $C_{81}H_{136}O_{17}P_2Na_2$: 3 linoleic, 1 oleic residue.

	Present work			Other work		Calculated	
	O_5b	O_7/16S	O_9/9S	*a*	*b*	*c*	*d*
P (%)	4·1	3·8	4·1	4·18	4·1	4·23	4·17
Glycerol (%)	19·1	17·3	19·3	17·5	—	16·80	18·6
Fatty acids (%)	—	74	75	73·0	—	76·6	75·4
Molar ratio ($P = 1·0$)							
Glycerol	1·57	1·53	1·56	1·40	1·5	1·34	1·5
Amino N	<0·03	0·08	<0·03	<0·03	<0·02	—	—
Inositol	Nil*	Nil*	Nil*	Nil*	Nil*	—	—

* Not detected.

structures of cardiolipin show the difficulty of determining the structure from the percentage composition.

Further work on the chromatographic purification is required. The method of fractionation on silicic acid is not entirely satisfactory. Cardiolipin is probably present as the acid in washed chloroform extracts and, even if the material is loaded on the column as the sodium salt, some material is eluted in a fast-running form free from cations; in this form, though the chemical composition is apparently unaltered, the solubility in ether and acetone and the serological activity progressively decrease (cf. Pangborn, 1944). This may be due to anhydride formation between neighbouring phosphoric acid groups, perhaps with some 'polymerization', for although a salt can be formed by immediate neutralization of the free acid it was impossible to make a salt from the 'polymerized' form. Moreover, although the cardiolipin band was clearly separated from the kephalin band, the cardiolipin still contained traces of nitrogenous and aldehyde derivatives, possibly because of occlusion.

The preparations of cardiolipin we obtained had a molar ratio of fatty acid ester to P of 2:1, but the molar ratio of glycerol to P was 3:2; this ratio of glycerol to P was also reported by Faure & Morelec-Coulon (1956). These results led us to suggest that cardiolipin contains three glycerol, two phosphoric acid and four fatty acid residues (structure V): in this structure, one glycerol residue is not fully esterified and the free hydroxyl has been assigned arbitrarily as a secondary alcoholic group on the middle glycerol. We tried to obtain evidence of structure in two ways (Macfarlane & Gray, 1957). First, an attempt was made to oxidize the postulated hydroxyl group of structure (V) with potassium permanganate and examine the acid hydrolysis products for the oxidized fragment. Secondly, an attempt was made to determine the ratio of glycerol to glycerophosphate formed by saponification and acid hydrolysis of cardiolipin, which was expected to be 1:3 on Pangborn's (1947) structure and 1:2 on structure (V). These experiments were inconclusive and led us to examine the nature of the products formed from cardiolipin in different hydrolysis conditions.

Hydrolysis products of cardiolipin after alkaline or acid saponification

The experiments were based on the following argument. Removal of the fatty acids from structure (V), or from Pangborn's structure with four glycerol residues, gives a water-soluble polyglycerophosphate. On treatment with periodate the terminal glycerol residues will be oxidized to (VI) (or an analogous structure). On acid hydrolysis (VI) can give glycolaldehyde phosphate (rapidly converted into orthophosphate), glycerol, glycerolmonophosphate, glycolaldehyde or glycerol diphosphate, according to the manner of splitting of the P diester bonds (1, 2, 3 and 4). If the intact cardiolipin is hydrolysed in conditions in which the rate of splitting of the fatty acid ester bonds is similar to that of the P diester bonds, a variety of mono- and di-glycerides, as well as glycerol, glycerol monophosphate and glycerol diphosphate, may be formed.

Samples of cardiolipin O_9/9S were saponified in different conditions, and the nature of the water-soluble phosphoric esters examined by determining

$$CH_2\cdot O\cdot R_1\cdot CH(OR_2)\cdot CH_2\cdot O\cdot PO(OH)\cdot O\cdot CH_2\cdot CH(OH)\cdot CH_2\cdot O\cdot PO(OH)\cdot O\cdot CH_2\cdot CH(OR_3)\cdot CH_2\cdot O\cdot R_4$$

(V)

$$\begin{array}{cccc} & (1)\downarrow & (2)\downarrow & (3)\downarrow \quad (4)\downarrow \\ OHC\cdot CH_2\cdot O\cdot PO(OH)\cdot O\cdot CH_2\cdot CH(OH)\cdot CH_2\cdot O\cdot PO(OH)\cdot O\cdot CH_2\cdot CHO & & & \end{array}$$

(VI)

(*a*) the proportion of phosphomonoester, by hydrolysis with a phosphomonoesterase preparation, (*b*) the apparent proportion of P present as α-glycerophosphate, by oxidation with periodate at room temperature, and estimating the orthophosphate formed subsequently in 1 hr. in N-H_2SO_4 at 100°, (*c*) the apparent amount of P as total glycerol phosphate, by estimating orthophosphate after oxidation with periodate at 100° in N-H_2SO_4 for 1 hr. (Burmaster, 1946). The conditions and results are shown in Table 7; all the P was recovered in the water-soluble fraction.

With mild alkaline conditions (Table 7A) the fatty acids are far more labile than the P diester linkages, and water-soluble polyglycerophosphate is formed. Only 40% of this appears as total glycerol phosphate by Burmaster's method. If the water-soluble polyester is first hydrolysed in N-HCl and then oxidized (A, ii) 100% appears as phosphomonoester, but only 88% as apparent glycerol phosphate.

By contrast, when the cardiolipin was completely saponified in acid (Table 7E) all the P appeared as phosphomonoesters, but only 60% appeared as glycerol phosphate by periodate oxidation and hydrolysis. In acid the lability of the P diester bonds with glycerol is apparently of the same order as that of the fatty acid linkages, for when a sample of cardiolipin was heated 10 min. in N-HCl at 100° only 60% of the P became water-soluble, and the fatty acid ester content of the ether-soluble portion was about 80% of the original. The presence of the substituent fatty acids appears to influence the manner of splitting of the P diester bands in acid in favour of formation of glycerol diphosphate, as only 60% of the P appeared as glycerol phosphate in E, compared with 88% in A ii. The formation of glycerol diphosphate was deduced from the results and has not been verified.

The kinetics of hydrolysis are clearly complex and may account in part for the inconclusive results previously reported. It is apparent that in analysis of material containing polyglycerol phosphatides, the estimation of 'glycerolphosphate P' by Burmaster's method may give misleading results.

Fatty acids present in cardiolipin

Two preparations of cardiolipin, $O_7/16S$, which still contained some nitrogenous impurity, and $O_9/9S$, which appeared to be free from kephalin and inositide, were saponified in N-NaOH at 20° for 4 hr. under N_2, and the fatty acids were extracted into ether, washed, dried, weighed and dissolved in methanol. Samples were taken for alkali isomerization and iodine values, and the remainder was converted into methyl esters which were examined chromatographically. The percentage composition of the mixed acids is shown in Table 8. The gas–liquid chromatograms did not distinguish satisfactorily between linoleic and linolenic acid but the sum of these acids agreed with the amounts calculated from the alkali-isomerization data; the amount of C_{20-22} acids were also in good agreement by the two methods, though the nature of the acids is not certain. The preparation $O_9/9S$ contained only 2·5% of saturated acids, while the less pure sample $O_7/16S$ contained about 8%; the percentage of saturated acids is perhaps as good a criterion of purity as any other, as the likely contaminants, kephalin and inositide, both contain about 50% of saturated acids. Palmitoleic and linolenic acids, as well as oleic acid, were present in the purest preparation $O_9/9S$, although, as found by Pangborn, the main acid present was linoleic. It is possible that cardiolipin, like the ester phosphatides, is a family rather than a single compound.

Table 7. *Nature of phosphorus esters formed from cardiolipin by hydrolysis in different conditions*

Conditions of saponification and hydrolysis	Nature of P esters (% of total)		
	Phosphomonoesters	Apparent glycerol monophosphate	
		α	α+β
A. Methanolic NaOH, 0·2N, 15 min. at 37°			
(i) Water-soluble product	0	21	40
(ii) After hydrolysis of (i) in N-HCl, 10 min. at 100°	100	40	88
B. Ethanolic N-NaOH, 2 hr. at 38°	62	—	—
C. 0·5N-KOH in 95% ethanol, 2 hr. refluxing			
(*a*) Water-soluble product	100	47	93
(*b*) After hydrolysis of (*a*) in N-HCl, 10 min. at 100°	100	87	91
D. Aq. N-NaOH			
(i) 2 hr. at 38°	40	—	—
(ii) 1 hr. at 100°	89	—	—
(iii) 2 hr. at 100°	100	46	91
E. N-HCl at 100° 2 hr.	100	42	60

Table 8. *Fatty acids of cardiolipin and glycerophosphoinositide*

(*a*) Gas–liquid chromatography by Dr A. T. James and Dr J. Webb. (*b*) Alkali-isomerization data.

Acid		Percentage of total fatty acids				
		Cardiolipin			Inositide	
		O_7/16S	O_8/9S			
(*a*)	(*b*)	(*a*)	(*a*)	(*b*)	(*a*)	(*b*)
C_{8-15}	—	1·12	Nil	—	Nil	—
n C_{16}	Palmitic	0·91	0·49	—	3·16	—
Branched unsaturated C_{17}	—	2·14	1·22	—	0·82	—
n C_{17}	—	—	—	—	0·73	—
n C_{18}	Stearic	2·67	0·79	—	45·95	—
Branched unsaturated C_{19}	—	0·96	—	—	—	—
Mono-unsaturated C_{16}	Palmitoleic	8·70	5·23	—	1·20	—
Mono-unsaturated C_{18}	Oleic	11·95	11·00	—	20·95	—
Diunsaturated C_{18}	Linoleic	59·50	80·00	70·8	12·26	7·63
Triunsaturated C_{18}	Linolenic	8·45		8·1		4·14
C_{20} (1)	Arachidonic	Tr.	0·74	1·24	10·78	10·94
(2)	C_{20-22} polyenoic	2·68	0·74	0·28	4·50	5·1
Total saturated acids		7·8	2·50	—	50·66	—
Total unsaturated acids		91·3	97·70	—	49·69	—

Glycerophosphoinositide

The acidic phospholipid fractions from methanol extracts of heart muscle (similar to fraction *A*, Table 2) contained inositol equivalent to about 20% of the P in the fraction. Attempts to crystallize inositide directly from this mixture in methanol, as described by Faure & Morelec-Coulon (1954), failed, but semicrystalline material apparently identical with the glycerophosphoinositide described by these authors was obtained from enriched fractions. On chromatographic fractionation of the acidic phospholipids of extract O_7 (p. 420) the rear fractions contained 50% of inositide. These fractions were dissolved as the sodium salt (55 mg. of P) in 50 ml. ether, and methanol was added (about 8·0 ml.) until the mixture was turbid; after 6 days at $-10°$ the semicrystalline deposit (161 mg.; P, 3·5; inositol, 16·8; glycerol, 12·0%) was collected by centrifuging and washed with ether, in which it was insoluble. Another sample was obtained from the acidic phospholipid fraction of extract O_8, after fractionation of the free acids with ethanol as described by Faure & Morelec-Coulon (1956); the ethanol-insoluble acids, containing kephalin and inositide and some cardiolipin, were redissolved in ether and the sodium salts (43 mg. of P) recovered by addition of sodium acetate and acetone; the ethereal solution (20 ml.) was treated with 2 vol. of methanol, and the mixture clarified by centrifuging: the supernatant, after several days at 2°, deposited semicrystalline material which was washed with ether, in which it was insoluble. Wt. 175 mg. (Found: P, 3·5; glycerol, 10·6; inositol, 19·9; fatty acids, 67%; $[\alpha]_D^{22}+7°$ in chloroform (*c*, 1·88). Calc. for stearyllinoleylglycerophosphoinositide; P, 3·5; glycerol, 10·4; inositol, 20·4; fatty acids, 64%). The saturated and unsaturated fatty acids were separated as the lead salts insoluble and soluble in 95% ethanol; the recovered fatty acids (55·5% saturated, iodine no. 30; 44·5% unsaturated, iodine no. 186) were examined by alkali isomerization; portions were also converted into methyl esters, which were analysed chromatographically. The results, calculated as a percentage of the total acids (Table 8), show that stearic acid accounts for nearly all the saturated acids, in agreement with Faure & Morelec-Coulon (1954); nearly one-third of the unsaturated acids were present as C_{20-22} polyenoic acids.

The yield of crystalline material from the acidic phospholipid fractions of O_7 and O_8 was only about 30% of the total inositide in the fractions and no more was obtained on concentration of the mother liquors. It is possible that the crystalline inositide is a 'stearic' subfamily. No evidence that the inositol was present in a form different from a glyceromonophosphoinositide was obtained.

DISCUSSION

The method of adsorption chromatography on silicic acid developed by Lea *et al.* (1955) has been applied in the present work to the separation on Mallinkrodt silicic acid of phospholipids in extracts from ox heart containing high proportions of kephalin and choline plasmalogens. This method can be used without breakdown of plasmalogens if the phospholipid mixture is largely free from other lipid material. In the presence of the neutral fat breakdown of plasmalogens, apparently limited to breakdown of kephalin plasmalogen, can occur, though this is minimized by ageing the column.

The entire fractionation of a total lipid extract, e.g. a chloroform–methanol extract, on silicic acid by a combination of the methods of Borgström (1952) and Lea *et al.* (1955) carries the risk of breakdown, though this can be detected by the formation of free aldehyde. The application of the method for the quantitative separation of intact tissue phospholipids is therefore dependent on a quantitative separation of the phospholipids from neutral fat. For preparative work, preliminary defatting with acetone and extraction with methanol reduced the risk of breakdown of plasmalogens though the extracted phospholipid may not be wholly representative.

The phospholipids present in washed chloroform–methanol extracts of ox heart were quantitatively recovered from silicic acid columns with good separation into four main classes: (1) nitrogen-free polyglycerophosphatides 9%; (2) serine and ethanolamine phosphatides 43%, of which about 45% was plasmalogen; (3) choline phosphatides 42%, of which 55% was choline plasmalogen and (4) sphingomyelin 5%. Inositides were not present in the washed chloroform–methanol extracts but were present in methanol extracts of heart in amounts equivalent to 2% of the total lipid P. No effective separation of the plasmalogen component from the corresponding ester phosphatide takes place on silicic acid. Cardiolipin was separated from the kephalin phosphatides, but the method is not entirely satisfactory; though preparations of cardiolipin which were serologically active were obtained the substance runs partly or wholly as the free acid, in which form some change in nature can take place, and it may appear in a number of fast-running bands. An outstanding problem is the clean separation of the serine and ethanolamine phosphatides, the glyceroglycerylphosphatides and the glycerophosphoinositides from each other.

Though it has limitations, chromatography on silicic acid is very useful for the separate assay of kephalin and choline plasmalogen; and by subsequent degradation of the separated kephalin and choline phosphatides with acetic acid, and separation on silicic acid of the free aldehyde, the lysophosphatide and the intact ester phosphatide, the fatty components of the plasmalogens and the ester phosphatides can be recovered separately.

In attempts to separate the intact plasmalogen from the corresponding ester phosphatide, the use of partition chromatography with secondary cellulose acetate as support was examined. With a *cyclo*hexane–methanol solvent system considerable enrichment was achieved, fractions containing up to 90% of plasmalogen being obtained. However, the iodine value indicated that members of plasmalogen and lecithin families had been separated according to the degree of unsaturation, and possibly also of chain length, of the constituent fatty acids. It appears that it would be very difficult to obtain pure plasmalogen which in its fatty components was representative of the whole content.

Simple phosphatidic acids, such as might be intermediates in the synthesis or metabolism of lecithin and kephalin, and lysolecithin were not detected in the ox heart extracts examined, and the presence of appreciable quantities of lysokephalin was traced to the breakdown of kephalin plasmalogen on the column. The plasmalogens appear to be stable in the excised tissue, which was not extracted with solvents till 2–3 hr. after death. The significance of the large proportions of choline and ethanolamine plasmalogens in heart is unknown. The differences in the fatty acids of the plasmalogens and the ester phosphatides found by Klenk & Krickau (1957) and Gray (1958*a*) and the characteristic fatty acid pattern of cardiolipin and glycerophosphoinositide focus attention also on the selectivity towards the fatty components involved in the biosynthesis of these phospholipids.

SUMMARY

1. Phospholipids equivalent to about 560 mg. of phosphorus/kg. were extracted from ox heart with chloroform–methanol mixtures and the washed lipid was fractionated on Mallinkrodt silicic acid columns.

2. The phospholipids, in mixtures which contained about 45% of plasmalogens but no inositides, were recovered quantitatively with good separation into cardiolipin, kephalin, lysokephalin, cholinephosphatides and sphingomyelin. The kephalin and choline phosphatide fractions contained about 45 and 55% respectively of plasmalogens which were not separated to any useful extent from the corresponding ester phosphatide.

3. In the presence of the neutral fat fraction, kephalin plasmalogen is liable to decompose on silicic acid columns, with formation of free aldehyde and lysokephalin. Apart from this decomposition, lysophosphatides were not detected.

4. On treatment of acetone-defatted heart with methanol, 95% of the choline phosphatides, together with cardiolipin and inositide, but only 70% of the kephalin was extracted.

5. A method for partition chromatography of phospholipids with organic solvents and secondary cellulose acetate as support is described. With *cyclo*hexane–methanol systems, fractionation of lecithin–choline plasmalogen mixtures containing 50% of plasmalogen yielded 20% of the total lipid in fractions containing 75–85% of plasmalogen. Evidence that the separation is influenced by the nature of the fatty acids was obtained.

6. Data on the composition of cardiolipin preparations and on the mode of hydrolysis of cardiolipin in acid and alkaline conditions are recorded.

7. Glycerophosphoinositide equivalent to about 30% of the inositide present was obtained as crystals from inositide-rich fractions. This inositide contained saturated and unsaturated acids in equimolar proportions: 46% of the fatty acids was present as stearic acid and 15% as C_{20-22} highly unsaturated acids.

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The Structure of the Plasmalogens of Ox Heart

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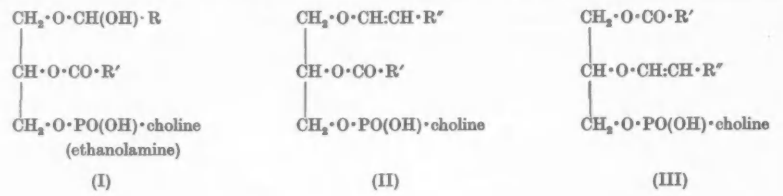
Although the natural plasmalogens have not yet been obtained as pure substances, the work of Klenk & Debuch (1954, 1955) and Debuch (1956) has shown conclusively that these plasmalogens contain a fatty acid as well as a fatty aldehyde residue. On treatment of fractions containing both the classical ester phosphatide and the plasmalogen with dilute acid to remove the aldehyde, lysolecithin was obtained from the choline-phosphatide fraction of ox-heart muscle (Klenk & Debuch, 1955) and lysokephalin from the fraction of brain (Debuch, 1956). Klenk & Debuch (1954) had earlier shown that after catalytic hydrogenation of the mixed kephalin fraction the aldehydic properties disappeared, and on alkaline or acid hydrolysis 60% of the phosphorus was present as chimyl or batyl alcohol phosphoric ester, i.e. as glyceryl ether derivatives. They suggested (I) or (II) as

probable structures for the plasmalogens in which the aldehyde was arbitrarily assigned to the α-position by analogy with the α-glyceryl ethers of fish oils. The position of the aldehyde was not determined.

Franzl & Rapport (1955) and Rapport & Franzl (1957) found that snake-venom phospholipase *A* removed fatty acid from a plasmalogen-rich lecithin fraction of heart as rapidly as from a pure lecithin prepared from egg yolk. They concluded that in choline plasmalogen the fatty acid must be in the α-position (III), as Hanahan (1954*a*) had shown that phospholipase *A* splits from lecithin only the α-acid. The action of phospholipase *A* on choline plasmalogen has been confirmed in this Laboratory, but although this hydrolysis is strong presumptive evidence that the aldehyde is on the β-carbon atom of the glycerol it is not a strict proof, for it has not been shown that the enzyme would not attack a β-acyl linkage if the α-atom were linked with an aldehyde.

The chemical proof of the β-aldehydo structure given in the present paper (Fig. 1) is based on the fact that choline plasmalogen treated with 90% acetic acid decomposes into free aldehyde and a lysolecithin which must be the β-acyl isomer (IV) if the aldehyde is originally linked on the α-carbon atom or the α-acyl isomer (VII) if the aldehyde is



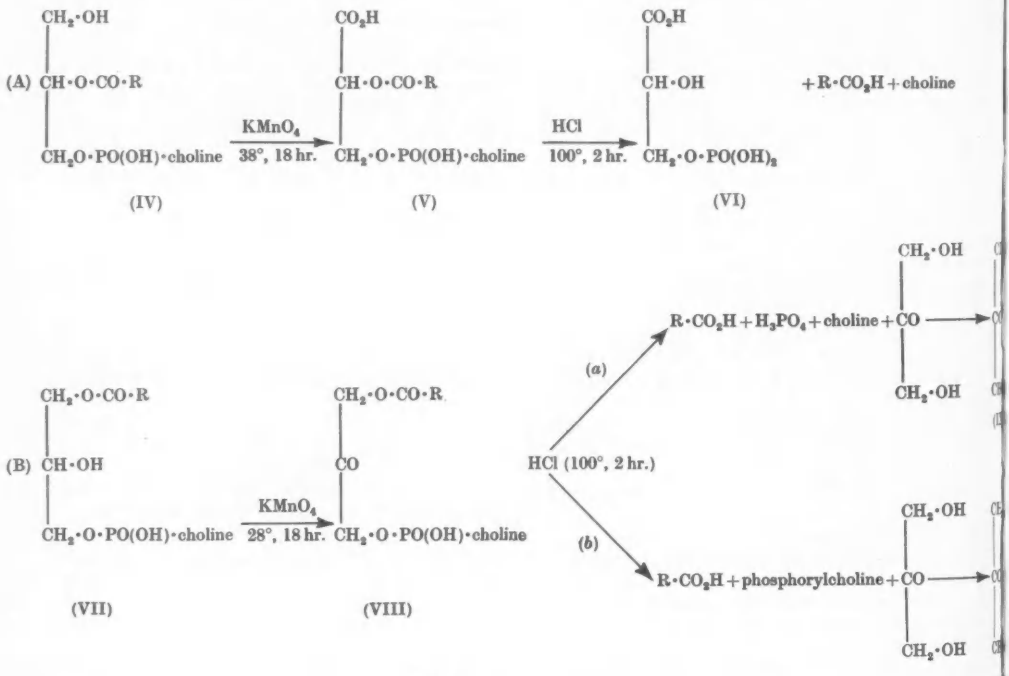


Fig. 1. Oxidation of α- and β-acyl-lysolecithins.

on the β-carbon atom. The β-acyl isomer, which is a known compound, usually obtained by the action of snake venom on lecithin, is oxidized by potassium permanganate to lysolecithinic acid (V), which on acid hydrolysis gives fatty acids, phosphoglyceric acid (VI) and choline (Hanahan, 1954*a*). Oxidation of the α-acyl-lysolecithin (VII), however, should produce a β-oxo compound (VIII), which on acid hydrolysis would give fatty acids and either (*a*) free choline and dihydroxyacetone phosphate, the latter being converted in acid into methylglyoxal (IX) and inorganic phosphate, or (*b*) phosphorylcholine and dihydroxyacetone, the latter giving methylglyoxal (IX). The hydrolysis products of (VII) are in either case different from those of (IV). The plan was to prepare the lysolecithin 'X' from plasmalogen and identify it as the α- or β-acyl isomer, by direct comparison of its properties with the known β-acyl isomer and by identification of the end products of permanganate oxidation and acid hydrolysis.

The experimental work entailed the preparation of the known β-acyl-lysolecithin as a control, and was extended to an analogous proof of the structure of ethanolamine plasmalogen. It also provided material for comparison of the constituent fatty acids of the plasmalogens and ester phosphatides of ox heart. A preliminary report of part of this work has been made (Gray, 1957).

EXPERIMENTAL

Methods. The analytical methods and general chromatographic procedures used and the preparation of the phospholipid fractions were described in the preceding paper (Gray & Macfarlane, 1958).

Position of the aldehyde group in choline plasmalogen

Preparation of lecithin and lysolecithin X. A typical choline-phosphatide fraction, a mixture of lecithin and choline plasmalogen, was obtained from ox-heart phospholipid by chromatography on silicic acid as described by Gray & Macfarlane (1958). The material (400 mg. of P; molar ratio P:choline, 1:1; plasmalogen value, 47%) was dissolved in 20 ml. of 90% (v/v) acetic acid and incubated at 38° for 18 hr. All the plasmalogen was split into free aldehyde and lysolecithin, but the breakdown of the lecithin, judged from the amount of water-soluble P formed, was negligible. The solution was diluted with 200 ml. of ether and shaken with sufficient NaOH solution to neutralize most of the excess of acid. The ether layer containing the lecithin, lysolecithin and aldehyde was shaken with several changes of water to remove the remaining acid; the combined aqueous layers contained 9·3 mg. P. Troublesome emulsions which formed sometimes during the washing were most readily broken by addition of chloroform and centrifuging. The solution of phospholipids and aldehydes was dried with Na_2SO_4 and the solvent was removed by vacuum distillation. The residue was dissolved in 95 ml. of methanol–chloroform (1:1, v/v); total P, 382 mg. The solution was chromatographed on a column containing 300 g. of silicic acid in methanol–chloroform (1:1, v/v) and this solvent was used for elution. Fractions of 25 ml. were collected automatically. The initial band quickly separated into three bands. The first (yellow) moved with the solvent front and contained all the free aldehyde and only 3 mg. of P. The two bands left were just visible by their slightly coloured fronts. As the fractions were collected, every second one was analysed for P and every fourth one for fatty acid ester groups. A typical separation curve is shown in Fig. 2. The sharpness of the separation, denoted by the decrease in the ratio of fatty acid ester to P from 2:1 at the peak of the second band (lecithin) down to 1:1 in the third band (lysolecithin), can be clearly seen. The fractions (total P, 363 mg.; recovery 94%) were pooled into two main fractions containing the lecithin and the lysolecithin X respectively and the intermediate fractions containing 20 mg. were discarded. The analyses of the main fractions are given in Table 1. The lecithin fraction was tested with the Feulgen reagent after hydrolysis in the usual test and gave no plasmal reaction. The recovery of the lysolecithin (P 163 mg.) was in good agreement with the amount expected from the original plasmalogen value.

Preparation of β-acyl-lysolecithin. The plasmal-free lecithin prepared above (1 g.; 40 mg. of P) was dissolved in 50 ml. of ether, and 2·2 mg. of mocassin venom (phospholipase *A*) in 0·5 ml. of 5 mM-$CaCl_2$ was added. A white precipitate of the β-acyl-lysolecithin formed as the reaction proceeded. After 4 hr. at room temp. the mixture was centrifuged, the supernatant removed and the precipitate washed several times with 10 ml. of anhydrous ether, dried under vacuum and dissolved in chloroform. The solution contained 39·2 mg. of P; the molar ratio P:fatty acid ester

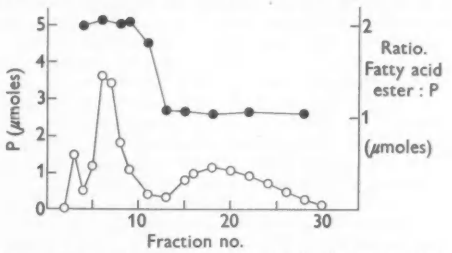


Fig. 2. Chromatographic separation of lecithin and α-acyl-lysolecithin. ○, Total phosphorus; ●, ratio of fatty acid ester groups to P. Adsorbent, silicic acid. Solvent, chloroform–methanol (1:1, v/v).

Table 1. *Analysis of lecithin and lysolecithin X separated on a silicic acid column*

	Lecithin	Lysolecithin X
Total P (mg.)	178	163
P (% on dry wt.)	4·15	5·9
N (% on dry wt.)	1·82	2·76
Molar ratio P:N	1:0·97	1:1·03
Molar ratio P:fatty acid ester	1:2·02	1:1·02
Double bond/mol. fatty acid ester	1·04	2·22

was 1:1·15, showing that the lysolecithin was contaminated with 15% of unchanged lecithin. This was removed by fractionating on silicic acid with methanol–chloroform 1:1 (v/v) as solvent; 30 mg. of P as pure β-acyl-lysolecithin was obtained.

Hydrogenation of lysolecithins. The lysolecithin X derived from plasmalogen and the β-acyl-lysolecithin were hydrogenated before oxidation. A sample of approximately 200 mg. (12·5 mg. of P) was dissolved in 10 ml. of dry chloroform–ethanol (1:4, v/v) and 15 mg. of platinum oxide (Adams catalyst) was added. The solution was shaken at room temp. for 2 hr. in hydrogen (1 atm.). On completion of the hydrogenation, which was tested by estimation of the iodine values, the catalyst was filtered off and the solutions were evaporated to dryness. The hydrogenated lysolecithins were less soluble in ethanol than the original lysolecithins and as solids were more powdery and less waxy.

Oxidation of hydrogenated β-acyl-lysolecithin to lysolecithinic acid. The solution of the hydrogenated β-acyl-lysolecithin was evaporated and the residue dissolved, readily, in 20 ml. of 0·1 M-sodium acetate buffer, pH 5·8 (Davidson, Long & Penny, 1956). Potassium permanganate (180 mg.) dissolved in 10 ml. of the buffer was added and after 18 hr. at 38° the mixture, which contained a large precipitate of MnO_2, was centrifuged. The magenta-coloured supernatant was removed and the precipitate washed three times with 15 ml. of hot water. The washings were added to the first supernatant and the excess of permanganate was destroyed with the minimum of sodium metabisulphite.

After removal of the MnO_2 by centrifuging, the colourless solution had a faint silky sheen and contained 11·7 mg. total P (94% recovery); orthophosphate, 2·3% of recovered P; molar ratio P to fatty acid ester 1:1. The solution was acidified to pH 1–2 with HCl and the precipitate was collected by centrifuging; a little more precipitate was obtained by adding an equal volume of acetone to the supernatant. The precipitates were combined, washed quickly with a little cold water, then with acetone and dried *in vacuo* to a white powder. This was dissolved in chloroform and analysed. A sample was also evaporated to dryness, dissolved in benzene–methanol (19:1, v/v) and titrated with 0·02N-sodium ethoxide. The molar ratio P : fatty acid ester : carboxylic acid found was 1:1·02:1·06, consistent with the structure of lysolecithinic acid (V).

Oxidation of hydrogenated lysolecithin X derived from plasmalogen. The hydrogenated material (11·5 mg. of P) was dissolved in 20 ml. of 0·1 M-acetate buffer, pH 5·8, giving a cloudy solution which cleared only on warming (cf. β-acyl-lysolecithin). Potassium permanganate (180 mg.) in 10 ml. of buffer was added and the mixture kept at 38° for 16 hr. It was then centrifuged and the precipitated MnO_2 washed with four 15 ml. portions of hot water. It was necessary to use hot water (above 60°) as the oxidized material was fairly insoluble in cold water and seemed to be held in some way by the MnO_2. The washings were combined with the original supernatant solution and the excess of permanganate destroyed by addition of the minimum amount of sodium metabisulphite. A further small precipiate of MnO_2 was removed by centrifuging. As the warm clear solution cooled to room temp. a considerable amount of flocculent white precipitate formed, which redissolved readily on warming. The solution contained 10·9 mg. of total P (95% recovery); inorganic P, 3% of recovered P; molar ratio P to fatty acid ester 1:1.

The solution was cooled to 0° and the precipitate which formed was removed by centrifuging. The supernatant solution was concentrated in a vacuum desiccator to half its volume and cooled in ice–water; an equal volume of ice-cold acetone was added to it and the precipitate which formed was added to the first one. This material, containing 9·1 mg. of P (79% recovery; a little more precipitate could be obtained by further concentration and extraction of the mother liquor), was dried under vacuum to a fine white powder, insoluble in ether, *cyclo*hexane, acetone and methanol, and fairly soluble in chloroform at room temperature but tending to come out of solution at 0°. P, 5·6% dry wt.; molar ratio P to fatty acid ester 1:1; no carboxylic acid group was present.

Acid hydrolysis of the oxidation products. Samples (about 1·0 mg. of P) of the lysolecithinic acid and the oxidation product X_0 of lysolecithin X were hydrolysed for 2 hr. at 100° in 2N-HCl. After cooling in ice–water the solidified fatty acids were removed either by filtration or extraction with ether. A small portion of each of the aqueous solutions was diluted with an equal volume of water and set aside for estimation of methylgloxal. The larger portions were neutralized with NaOH solution and samples were analysed for total and inorganic P and free choline. The results in Table 2 show that only very small amounts of free choline and orthophosphate were produced from X_0, while lysolecithinic acid gave as expected the calculated amount of choline as free choline. As the oxidation products were different, the lysolecithin X must have been the β-acyl isomer VII.

Identification of P-containing products of hydrolysis. Samples of the neutralized hydrolysis products, together with blanks, were incubated with bone phosphatase free from diesterase, in bicarbonate buffer as veronal interferes with the choline determination. After 1 hr. at 38°, pH 8·6, 2·0 ml. of 10% trichloroacetic acid was added and the acid filtrate analysed for orthophosphate and free choline. Table 3 shows that the P-containing product of acid hydro-

Table 2. *Products of acid hydrolysis of lysolecithinic acid and* X_0

All results are given in μmoles.

	Lysolecithinic acid	X_0
Total P	2·16	2·98
Total choline*	2·16	2·98
Inorganic P	0·162	0·164
Free choline	2·14	0·208†

* Calc. from total P.
† Corrected from observed value for presence of excess of phosphorylcholine (Webster, 1956).

Table 3. *Hydrolysis with phosphatase of the acid-hydrolysis products of lysolecithinic acid and* X_0

Samples (0·5 ml.) + 1·0 ml. of $NaHCO_3$–Na_2CO_3 buffer, pH 8·6 + 0·2 ml. of phosphatase + 0·3 ml. of water; 40 min. at 38°. All results are given in μmoles.

	Lysolecithinic acid	X_0
Total P	0·855	1·32
Total choline (calc.)	0·855	1·32
Inorganic P	0·85	1·35
Free choline	0·84*	1·36

* Choline already freed by previous acid hydrolysis.

lysis of X_0 was phosphorylcholine, as the total P present and the equivalent amount of choline were found as orthophosphate and free choline after the action of the phosphomonoesterase. The phosphoglyceric acid in the hydrolysed solution of lysolecithinic acid (which already contained free choline) was hydrolysed by the enzyme, as expected. The small amounts of inorganic P (6–8%) found in the acid hydrolysis products (Table 2) were consistent with amounts expected from the hydrolysis of phosphoglyceric acid or phosphorylcholine in the conditions (2 hr. in 2N-HCl at 100°).

Another sample of lysolecithinic acid (P 6 mg.) was hydrolysed with acid as above and the phosphoglyceric acid present in the hydrolysed solution was isolated by concentrating 5 ml. to 2 ml., acidifying with 0·5 ml. of 5N-HCl and adding excess of $BaCl_2$ followed by 1 vol. of ethanol. The acid Ba salt was purified twice by dissolving in 0·5 ml. of N-HCl and reprecipitating with 1 vol. of ethanol; the final precipitate was washed with ethanol and dried over P_2O_5. The yield was about 60% (50 mg.) of the theoretical. This phosphoglyceric acid was optically inactive in presence of 8% ammonium molybdate solution ($[\alpha]_D$ found for a sample of 3-D-phosphoglyceric acid in molybdate was $-360°$).

Formation of methylglyoxal by acid hydrolysis of X_0. The small portions of the acid-hydrolysed solutions of lysolecithinic acid and X_0 set aside previously were tested for methylglyoxal by the method of Ariyama (1928). The solutions were neutralized with NaOH just before testing. Samples of 0·5 ml. together with a blank containing water and a standard containing 0·25μmole of methylglyoxal were placed in tubes and to each tube 0·2 ml. of Benedict's uric acid reagent (phosphoarsenotungstate), 0·1 ml. of M-KCN and 0·3 ml. of M-Na_2CO_3 were added in that order. The hydrolysed X_0 sample gave a deep-blue colour; the amount was about 45% of that expected on the P content. However, if methylglyoxal is heated in HCl with traces of manganese (which were still present in X_0) the colour yield is decreased. The blanks and the hydrolysis products from the lysolecithinic acid gave no colour with the reagent.

Conclusion. The oxidation products of the lysolecithin derived from choline plasmalogen by removal of the aldehyde chain is the β-oxo compound (VIII), differing from the oxidation product (lysolecithinic acid, V) of β-acyllysolecithin (IV) in that on acid hydrolysis it gives phosphorylcholine and methylglyoxal, instead of phosphoglyceric acid and free choline. The original plasmalogen had therefore the α-acyl-β-aldehydo structure (III).

Position of the aldehyde group in ethanolamine plasmalogen

The method used to prove the position of the aldehyde group in ethanolamine plasmalogen was the same as that used for choline plasmalogen, i.e. by identification of the lysokephalin formed by the action of weak acid on the plasmalogen as the α-acyl isomer. This was done by oxidation with permanganate and identification of the hydrolysis products. The procedure was complicated by the necessity of protecting the amino group by acetylation before oxidation.

Preparation of phosphatidylethanolamine and lysokephalin X. A typical kephalin fraction from ox heart (349 mg. of P), containing 33% of the P as ethanolamine plasmalogen, was dissolved in 12 ml. of 90% (v/v) acetic acid and kept at 38° overnight. The phospholipids and aldehyde were extracted into ether and the ethereal solution was well washed with water as before, dried and evaporated. The residue was taken up in methanol–chloroform (1:49, v/v) and the solution was chromatographed on 300 g. of silicic acid in the same solvent, which was used for elution. The free aldehyde passed rapidly through the column and when the eluate gave no reaction for aldehyde (by spot test with $HgCl_2$ and the Feulgen reagent) the solvent was changed to methanol–chloroform (1:4, v/v). The brown band of phospholipid at the top of the column then separated into a fast-moving brown band and a very slow-moving yellow band. After elution of the brown band the solvent was changed to methanol–chloroform (2:3, v/v) and the yellow fraction was eluted. The analyses (Table 4) showed that the brown and yellow fractions contained the kephalin and the lysokephalin X respectively.

The lysokephalin fraction was evaporated to dryness, dissolved in a little chloroform and cooled in ice, and ice-cold ether was added. The white precipitate was separated and washed with ether by centrifuging, dried under vacuum and dissolved in chloroform to give a very pale yellow solution, total P 97 mg. The recovery of purified lysokephalin, based on the plasmalogen value of the original mixture, was 84%, and of the phosphatidylethanolamine 92%.

Acetylation of the amino group of the lysokephalin. As the hydroxyl group on the glycerol moiety had to be left free, the selective method of White (1940) for *N*-acetylation was used. Approximately 200 mg. (0·44 m-mole) of lysokephalin X (12 mg. of P) was dissolved in 2·0 ml. of dry methanol; 70 mg. (0·42 m-mole) of silver acetate and 57 mg. (0·52 m-mole) of acetic anhydride were added and the flask was shaken at room temp. for 3·5 hr. The solution was refluxed for 5 min. and filtered hot. The residue was washed with a little methanol and the washings added to the main solution. Analysis for P and amino N showed that acetylation was 85% complete. The solution was taken to dryness and the residue dissolved in 2·0 ml. of methanol–chloroform (1:9, v/v), and chromatographed on 4·5 g. of silicic acid in this solvent. The *N*-acetylated lysokephalin was quickly eluted in the first few fractions, followed by the lysokephalin. The solvent was evaporated from the *N*-acetylated material, leaving a straw-coloured syrup which was dissolved in 4·0 ml. of chloroform (total P, 10·0 mg.; amino N, less than 2% of total).

Hydrogenation of N-acetyl-lysokephalin X. The material (9 mg. of P) was dissolved in 15 ml. of chloroform–ethanol (1:2, v/v) and shaken with 8 mg. of platinum oxide (Adams catalyst) for 2 hr. in hydrogen at 1 atmosphere and room temp. After filtration the solution contained 9 mg. of

Table 4. *Analysis of kephalin and lysokephalin X separated on a silicic acid column*

	Kephalin	Lysokephalin X
Total P (mg.)	216	97
P (% on dry wt.)	4·1	6·4
N (% on dry wt.)	1·88	2·96
Amino N/total N (%)	106	102
Molar ratio P:N	1:1·01	1:1·02
Molar ratio P:fatty acid ester	1:2·01	1:1·0
I_2 value (double bonds/mol. fatty acid)	1·79	3·2

P; molar ratio P to fatty acid ester, 1:1; iodine value, nil; amino N/total N, less than 2%.

Oxidation of hydrogenated material. The material was evaporated to dryness (8·8 mg. of P) and 15 ml. of 0·1M-acetate buffer (pH 5·6) was added, giving a white emulsion. Potassium permanganate (150 mg.) was added and the mixture was kept at 38° for 18 hr. It was then centrifuged and the supernatant solution (7·5 mg. of P) removed. The precipitate was washed twice with hot water, which recovered 1·3 mg. more of P. The first supernatant was cooled in ice and an equal volume of ice-cold acetone was added; the flocculent precipitate was recovered by centrifuging and dried under vacuum, to a fine white non-sticky powder. The acetone supernatant was concentrated and a further amount of precipitate recovered on addition of acetone. The combined precipitates were dissolved in chloroform and a small amount of inorganic salt removed by centrifuging; the solution of the oxidation products contained 6·8 mg. of P (recovery 91%).

Acid hydrolysis of the oxidation product of N-acetyl-lysokephalin. Two samples of the oxidized material (1·0 mg. of P) were hydrolysed, one in 2N- and one in 5N-HCl for 2 hr. at 100°. The solutions were cooled in ice–water, filtered and neutralized. After 2 hr. in 2N-HCl, 85% of the amino group was deacetylated and 21% of the P was orthophosphate. (By contrast, when a sample of the lysokephalin was oxidized without acetylation, and then hydrolysed 2 hr. in 2N-HCl, only 9% of the P was converted into orthophosphate, but some breakdown of the amino group occurred.) After 2 hr. in 5N-HCl deacetylation was complete (molar ratio P:amino N, 1:1·05).

The neutralized hydrolysis product was tested for methylglyoxal, with positive results; the amount was only about 50% of that expected on the P content. All the P was present as a phosphomonoester, estimated enzymically. The remaining solution was treated with $BaCl_2$; the precipitate contained only inorganic phosphate. The filtered solution was then treated with an equal volume of ethanol, but no precipitate formed. It was concluded from the absence of any insoluble barium salt that no phosphoglyceric acid was present, and from the presence of a very soluble phosphomonoester and methylglyoxal that the hydrolysis products were phosphorylethanolamine and methylglyoxal, together with fatty acids and acetic acid. The oxidation product was therefore the *N*-acetyl-β-oxo-lysokephalin, analogous to (VIII). The original plasmalogen therefore had the α-acyl-β-aldehydo structure, giving the α-acyl-lysokephalin on removal of the aldehyde.

Nature of the aldehydes in the choline plasmalogen of heart muscle

The aldehydes were isolated quantitatively from lecithin–choline plasmalogen and kephalin–ethanolamine plasmalogen fractions as their dimethylacetal derivatives and tested for unsaturation. The iodine values (equivalent to 0·12 and 0·17 double bond/aldehyde for choline plasmalogen and ethanolamine plasmalogen respectively) showed that most of the aldehydes were saturated.

Isolation as the 2:4-dinitrophenylhydrazone derivatives. A typical lecithin–choline plasmalogen mixture was treated with 90% acetic acid at 38° for 18 hr., and the free aldehydes were separated as described above (p. 427) from the residual phospholipids on a silicic acid column. The chloroform solution of the aldehydes was evaporated to dryness and the residue dissolved in dry ethanol. The 2:4-dinitrophenylhydrazones were prepared (Allen, 1930) and recrystallized from hot ethanol.

Fractionation of the dinitrophenylhydrazone. The method of Kramer & Van Duin (1954) for chromatographic fractionation was tested with an artificial mixture of palmitaldehyde and stearaldehyde 2:4-dinitrophenylhydrazones, which were well separated. A sample of the plasmalogen derivatives (0·5 mg.) was put on a silicic acid column [15 g., prepared by Isherwood's (1946) method] mixed with 16 ml. of nitromethane; height 29 cm., diameter 1·1 cm. The column was developed with light petroleum (b.p. 40–60°) equilibrated with nitromethane. The band separated into two bands corresponding in position to those of the stearal and palmital derivatives, the rear band (palmital) being obviously larger. The bands were collected separately and the material dissolved in equal volumes of chloroform. By comparison of the colour value in the EEL colorimeter, filter 621, the second band was approximately four times as concentrated as the first. The results indicated that the aldehydes of choline plasmalogen consisted of about 80% of palmitaldehyde and 20% of stearaldehyde, but the method did not exclude the presence of traces of unsaturated aldehydes or of aldehydes of odd-numbered chain length.

Gas-phase chromatography. The mixed aldehydes were examined as their dimethylacetal derivatives and synthetic palmitaldehyde dimethylacetal was used as a reference compound. This 'pure' standard was found to be only 77·5% of palmitaldehyde with three closely associated aldehydes (4·4, 11·25 and 4·35%) as the main impurities. These impurities, however, would make little if any detectable difference in the Feulgen colour reaction of this standard from the colour given by 100% palmitaldehyde.

The mixture of the natural aldehydes was shown to contain 41% of palmitaldehyde (the major component) with two lower aldehydes (16 and 9·5%, identical with two of the 'impurities' in the palmitaldehyde standard) making up 66·5% of the total. The remaining 33·5% was distributed between 15 aldehydes; seven of these, one of which was stearaldehyde, were of higher molecular weight than palmitaldehyde, and accounted for 19% of the total aldehydes. The amount of stearaldehyde present appeared to be less than 5%.

Fatty acids of the plasmalogens

During the fractionation of lecithin–choline plasmalogen mixtures by partition chromatography (Gray & Macfarlane, 1958) it was observed that the iodine value of the front fractions rich in choline plasmalogen was greater than that of the back fractions, indicating that the fatty acids in the

Table 5. *Unsaturation of fatty acids in phospholipids of heart muscle*

Phospholipids	Double bonds/fatty acid*
Lecithin	1·04
β-Acyl-lysolecithin	0·34
α-Acyl-lysolecithin†	2·20
Kephalin	1·79
α-Acyl-lysokephalin‡	3·20

* μmoles of iodine taken up per μmole of fatty acid ester.
† From choline plasmalogen.
‡ From ethanolamine plasmalogen.

plasmalogens were more unsaturated. This fact was confirmed by estimation of the iodine values of the pure lecithin and kephalin, the pure α- and β-acyl-lysolecithins and the α-acyl-lysokephalin prepared during the present work. The results in Table 5 show that the average number of double bonds/mol. of fatty acid is higher in the plasmalogen derivatives (one fatty acid) than in the ester phosphatides, but the interpretation is not clear unless the proportion of saturated and unsaturated acids is known. The compounds were therefore saponified and the fatty acids fractionated quantitatively by lead salts, and reanalysed.

Samples of the phospholipids (100–200 mg.) were saponified in 6·0 ml. of aqueous N-NaOH in a stoppered tube filled with N_2, at room temp. overnight. The mixtures were warmed at 45° for 15 min. to liquefy them and acidified with 4·0 ml. of 5N-HCl. The fatty acids were extracted with ether, and the ethereal solution washed with water and evaporated. The acids were dissolved in 1·5 ml. of 95% ethanol, and 5·0 ml. of 95% ethanol containing 100 mg. of lead acetate was added at boiling point. The mixture was allowed to cool slowly overnight, and the insoluble lead salts were collected by centrifuging and washed three times with 2·0 ml. of 95% ethanol. The washings were added to the soluble fraction, which was evaporated. The fatty acids were recovered by decomposing the insoluble and soluble lead salts with 2N-HNO_3 and 2N-HCl respectively, ethanol solution being washed with water and dried by repeated evaporation with benzene. After weighing, the acids were dissolved in methanol, and samples taken for iodine values (Trappe, 1938) and alkali isomerization (Herb & Riemenschneider, 1953).

Table 6 shows that slightly more than half of the fatty acids in the ester phosphatides and nearly all the acids in the α-acyl lyso compounds derived from plasmalogen were unsaturated. The degree of unsaturation was very much higher in the ethanolamine derivatives than in the choline derivatives. Table 7 shows the percentage composition of the unsaturated fatty acids based on the absorption at 233, 268, 315 and 346 mμ after alkali isomerization, from which values ascribed to linoleic acid, linolenic acid, arachidonic acid and C_{20-22} polyenoic acids were calculated, the remainder calculated by difference being ascribed to oleic acid. The unsaturated acids in the lecithin and kephalin were slightly more than 50% of the total fatty acids. It is usually considered that the unsaturated acids occur on the α-position. The β-acyl-lysolecithin had in fact a very low iodine value (Table 5). The unsaturated acids shown in Table 7 therefore probably substantially make up the α-acyl component of the lecithin, choline plasmalogen, kephalin and ethanolamine plasmalogen of ox heart respectively.

DISCUSSION

The plasmalogens and the ester phosphatides used in this study were representative of the complete range present in ox-heart phosphatides, because the quantitative extraction and fractionation of the phosphatides was followed by hydrolysis of the whole lecithin and kephalin fractions obtained. The results show that the aldehyde residue is apparently exclusively linked to the β-carbon atom of the glycerol in the plasmalogen molecule. This confirms

Table 6. *Percentage and iodine number of saturated and unsaturated acids of phospholipids*

Fatty acids were separated as the insoluble lead salts.

	Source of fatty acids			
	Lecithin	α-Acyl-lysolecithin*	Kephalin	α-Acyl-lysokephalin†
	Saturated fatty acids			
Wt. (mg.)	26·7	3·6	43·7	7·7
% of total	43·0	3·5	46·0	7·0
Iodine no.	8	89	11	55
	Unsaturated fatty acids			
Wt. (mg.)	35·0	97·7	51·5	102·2
% of total	57·0	96·5	54·0	93·0
Iodine no.	153	187	269	260

* From choline plasmalogen. † From ethanolamine plasmalogen.

Table 7. *Composition of unsaturated fatty acids of phospholipids by alkali isomerization*

	Percentage of total unsaturated acids			
Acid	Lecithin	α-Acyl-lysolecithin*	Kephalin	α-Acyl-lysokephalin†
Oleic‡	55·2	40·0	33·0	18·6
Linoleic	32·0	39·0	13·6	35·0
Linolenic	7·3	8·0	1·5	11·0
Arachidonic	5·2	10·0	41·5	23·0
C_{20} polyene	0·3	3·0	10·4	12·4

* From choline plasmalogen. † From ethanolamine plasmalogen. ‡ By difference.

the conclusions reached by Rapport & Franzl (1957) from the action of phospholipase *A* on choline plasmalogen of ox heart and shows that the ethanolamine plasmalogen has a similar structure.

In ox heart almost all the aldehydes isolated from the plasmalogens were saturated compounds. Gas-phase chromatography confirmed that palmitaldehyde was the major component of the aldehydes isolated from choline plasmalogen, and showed the presence of seventeen other components, fifteen of them occurring in amounts of only 1–5%. This sensitive analytical procedure illustrates very clearly the complexity of the natural plasmalogens.

The fatty acids in the plasmalogens were found to be mainly (95%) unsaturated acids. As a result of the work of Hanahan (1954*b*, *c*) and Rhodes & Lea (1956) with phospholipase *A* it was shown that in lecithin and kephalin the α-position was mainly occupied by unsaturated fatty acids and the β-position mainly by saturated fatty acids. This general structural arrangement was found in phosphatides from various sources (liver, brain, egg yolk). The positions of the saturated and unsaturated aliphatic chains in the plasmalogens of ox-heart are the same as those in the ester phosphatides. There is therefore a very close similarity of structure between these two classes of compounds (cf. also Long & Penny, 1957).

Marinetti & Erbland (1957) reported that the plasmalogens isolated from pig heart had the aldehyde residue linked exclusively in the α-position. Their method of proof was different from that described in this paper and involved a periodate-oxidation procedure and infrared measurement. Their results imply that the plasmalogens from different species of animals may have different structures. If there is as close a relationship between the plasmalogens and ester phosphatides of pig heart as there is in ox heart one would expect a corresponding difference in the positions of the saturated and unsaturated fatty acids in the ester phosphatides. A systematic investigation of various tissues of different species of animal is needed to obtain a clearer picture of plasmalogen structure and composition.

Kephalin and ethanolamine plasmalogen contain a higher proportion of the highly unsaturated acids, arachidonic acid and C_{20} polyethenoic acids than do choline plasmalogen or lecithin. Phosphatidylethanolamine from egg is also almost twice as unsaturated as phosphatidylcholine from the same source (Rhodes, 1957), and a similar difference appears in the kephalin and lecithin fractions from human brain (Klenk, Debuch & Daun, 1953; Debuch, 1956).

The compositions of the unsaturated fatty acids from choline plasmalogen determined by alkali isomerization (Table 7) are in substantial agreement with the results of Klenk & Krickau (1957). A comparison of the unsaturated acids from lecithin and choline plasmalogen (Table 7) shows a higher degree of unsaturation in the plasmalogen arising from the greater amount of linoleic, arachidonic and C_{20} polyethenoic acids. The ethanolamine plasmalogen, however, does not show a general increase in unsaturation over kephalin, because the larger amounts of linoleic acid and linolenic acid in the plasmalogens are offset by the very large amount of arachidonic acid in the kephalin.

SUMMARY

1. The lysolecithin derived from the choline plasmalogen of ox heart by removal of the aldehyde residue was identified as the α-acyl isomer by oxidation with permanganate and identification of the acid hydrolysis products.

2. The lysokephalin similarly derived from kephalin plasmalogen was identified as α-acyllysokephalin.

3. The aldehyde residue in the plasmalogens of ox heart is therefore linked to the β-carbon atom of the glycerol moiety.

4. The aldehydes isolated from the choline plasmalogen were mainly saturated, the major component being palmitaldehyde. The fatty acids present in choline and ethanolamine plasmalogen were mainly (95%) unsaturated acids.

5. The composition of the unsaturated fatty acids of the plasmalogens and of the kephalin and lecithin of ox heart was examined by alkali isomerization. The ethanolamine phosphatides were more highly unsaturated than the choline phosphatides, and contained large amounts of C_{20-22} polyenoic acids.

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Electrophoresis in a Density Gradient

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In the method of zone electrophoresis a means for the stabilization of the medium against convection has to be provided. That a density gradient of sucrose might be suitable for this purpose was suggested by Consden, Gordon & Martin (1946). Since then several attempts, discussed by Svensson, Hagdahl & Lerner (1957), have been made to achieve separations on this principle. In such systems it is comparatively easy to obtain good separations of substances if they are present in such small amounts as not to contribute significantly to the density of the initial solution, or if they are of approximately the same molecular size as the substance used for making the gradient. These conditions can be met quite satisfactorily with dyestuffs in a sucrose gradient, but not so easily with proteins. With large molecules, instability of the initial zone develops rapidly, even though its density has been adjusted so that it is slightly less than that of the solution on which it rests. This happens because the decrease in density due to diffusion of large molecules out of the zone is more than compensated by the more rapid diffusion of sucrose into the zone. As a result, the zone becomes relatively dense and droplets are formed. Fortunately this difficulty has now been overcome by Svensson *et al.* (1957), who have shown that droplet formation in the initial zone does not occur if an appropriate amount of a dense, rapidly diffusing substance such as potassium bromide is present in the protein solution. Most of Svensson's work was carried out either with dyestuffs (Svensson & Valmet, 1955) or with normal or pathological sera (Svensson *et al.* 1957). The aim of the present work has been to examine the reproducibility and completeness of the separation of proteins in a modification of the apparatus used by Svensson *et al.* (1957). For this purpose human albumin and artificial mixtures of human albumin and haemoglobin have been used.

Complete separation of many of the proteins of human and animal sera can, of course, be achieved readily by the method of zone electrophoresis described by Porath (1954). Probably the most serious disadvantage of this method is the adsorption of traces of the proteins on the cellulose matrix of the column. Evidently the sucrose-gradient column avoids all difficulties due to adsorption, except on the glass of the apparatus. Since the possibility of adsorption of virus particles on a solid matrix cannot be ignored, it is interesting to note that Cramer, Lerner & Polson (1957) have recently employed sucrose-gradient electrophoresis for a problem of this kind. We report here an application to the slow-reacting substance A of Brocklehurst (1955, 1956), which possesses the property of stimulating guinea-pig gut.

EXPERIMENTAL

The apparatus (Fig. 1), basically the same as that of Svensson *et al.* (1957), incorporates some novel features. It was constructed with all-glass joints, held together by springs. The joints at the top of the electrode vessels were inverted to eliminate accumulation of large gas bubbles, which otherwise were periodically detached during electrophoresis, producing slight but undesirable movement of the liquid. Filling of the gradient tube was made automatic. The liquid from the mixer was fed through a long capillary tube which was held in contact with the wall of the column about an inch above the meniscus. This capillary was raised intermittently by means of a screwed-rod mechanism actuated by two contacts attached to the capillary tube and touching the liquid surface. Virtually no current passed between the contacts, since the control was exercised through a sensitive electronic relay.

With sucrose columns, sections 2 and 4 (Fig. 1) were first filled with plain buffer. Next the capillary tube 7 was

filled with buffer containing 50% (w/v) of sucrose (by suction), which was also used to fill vessel 8 to a height of several inches, and column 6 to a few centimetres below the top of the capillary. The rest of vessel 8 was filled with plain buffer. After the gradient had been produced in 6, the apparatus was filled to the top with plain buffer, and thus completely filled with liquid. Two or three 10 ml. samples were then withdrawn from tap 7 by siphoning buffer in through tap 3. The sample, its density adjusted with the help of an auxiliary oil gradient, was siphoned in via tap 7.

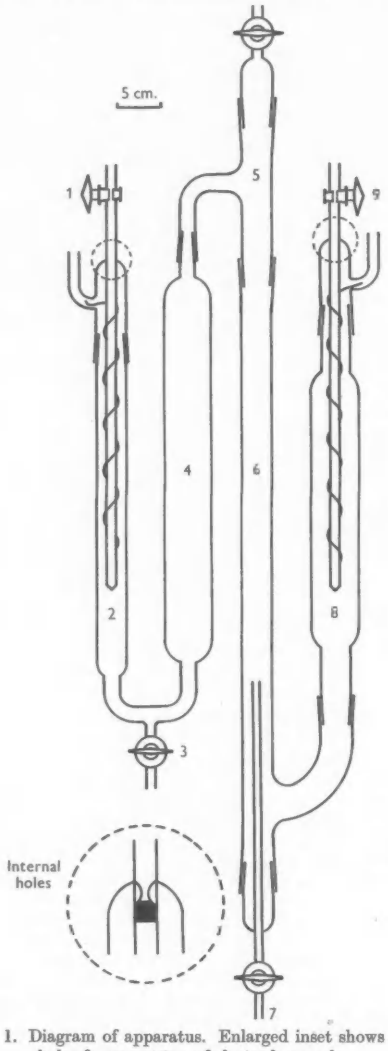

Fig. 1. Diagram of apparatus. Enlarged inset shows exit holes for gas at top of electrode vessels.

The potassium bromide concentration (w/v) was made equal to that of the protein, as this condition prevented droplet formation. Generally approx. 3 ml. of sample was introduced in 20–30 min., producing a zone about 5 mm. high. To minimize movement of the initial zone, levelling was carried out by adjusting the heights of the menisci in the narrow tubes at the top of 1 and 9 before these taps were opened. The difference in the levels of the menisci, to compensate for the gradient column, was usually about 15 and 5 mm. for sucrose and ethanol respectively. The meniscus on each side was then lowered by the same distance (with the appropriate tap open) in order to bring the levels to wider regions of 1 and 9 before taps 1 and 9 were both finally opened.

With ethanol gradients, the whole apparatus was filled with plain buffer, except for the column, and for the top of vessel 4 and the region 5, which contained buffer mixed with ethanol. Theoretically it would be preferable for the aqueous buffer in the mixer to be free of air, but in practice most of the air expelled on mixing with the 50% (w/v) ethanol is eliminated as the solution flows out of the capillary tube feeding in the gradient. The auxiliary oil gradient proved unsuitable, owing to loss of ethanol from the drops to the column. Densities were, therefore, adjusted by means of floats, each of which consisted of a small piece of polythene sheet transfixed by a short length of Nichrome wire (the particular polythene used gave floats of about 8 mm. diameter each carrying 2–3 mm. of 0·27 mm. diameter wire). The mean density of these floats could be increased when necessary by clipping off small sections of the polythene. It was essential, of course, to prevent air bubbles sticking to the floats. The potential difference applied to the apparatus, 450–550 v, produced an initial current of 20–27 mA, which dropped by 20–30% during 24 hr. and more rapidly subsequently, unless the buffer in the left-hand electrode vessel was renewed. When this was done, the final drop over 48 hr. was about 30% of the initial value.

At the conclusion of the run, 2–3 ml. fractions were run out at the rate of 1–2 ml./min. These were used for measurements of visible light-absorption at 540 mμ (with haemoglobin) or ultraviolet absorption at 279 mμ (with albumin and insulin) in the Beckman DU spectrophotometer. In the most accurate work, corrections were applied for the absorption of the sucrose (or possibly impurities in it) after the sucrose concentration in various tubes had been estimated by means of dipping refractometer measurements on samples diluted by weight. The shape of the sucrose gradient was nearly, but not quite, linear at the end of the run. The A.R. sucrose used in all experiments gave blank readings approximately 50% of those of ordinary sucrose. In sucrose gradients, 0·025 M-borax (pH 9·2) was used, as recommended by Svensson *et al.* (1957). In ethanol gradients 0·025 M-ammonium acetate was used, adjusted by means of ammonia to pH 8 or 9. Neither of these buffers absorbed significantly at 279 mμ.

The experiments illustrated by Figs. 2–4 were done with human albumin (sample *A*) used in other work (Charlwood, 1954). Another sample used was prepared by column electrophoresis (Porath, 1954). Haemoglobin was prepared from washed human red cells by haemolysis with water and toluene, followed by high-speed centrifuging to remove the stroma. The crystalline zinc insulin (batch number 9333/1404) was supplied by Burroughs Wellcome and Co.

Iodinations were carried out by the method of McFarlane (1956), to the extent of a mean substitution of not more than 1 atom of total iodine/mol. In most cases the albumin was subsequently diluted with several times its weight of untreated albumin. Radioactivities of iodinated samples were measured on weighed samples in a scintillation counter, and corrected to a volume basis. Error due to the counter was never more than 3% and generally better than 1%.

The slow-reacting substance was prepared from perfused guinea-pig lungs and partially purified by Mr W. E. Brocklehurst as described previously (Brocklehurst, 1955). This material, of unknown composition, was estimated by its ability to cause contraction of the isolated guinea-pig ileum. Gradient electrophoresis experiments were done with samples containing 2000–9000 effective doses, at which levels the weight of the material introduced was small (<5 mg.).

RESULTS

These have mostly been plotted as curves, although strictly histograms should be used. The reproducibility of the technique is shown by the results of duplicate runs on human albumin (Fig. 2). The points shown, one set obtained with the use of a fraction collector, the other by manual collection of fractions, all lie virtually on the same curve. They are uncorrected for the solvent background, but the magnitudes of these corrections are shown by the triangles in Fig. 2. It is thus clear that tubes before number 25 contained no detectable protein.

Fig. 3 shows results of an experiment designed to study the quality of separations. The two proteins haemoglobin and albumin were located by entirely independent methods, the former by absorption in the visible region and the other by radioactivity measurements of the ^{131}I incorporated in it. Evidence of freedom from mutual contamination was obtained by reciprocal measurements, i.e. no radioactivity could be found in the haemoglobin, nor light-absorption at 540 mμ in the albumin solutions.

Fig. 4 shows the distribution of optical density and radioactivity in a typical albumin run. The peak of activity in different experiments moved consistently just ahead of that of optical density. This is shown clearly by the curve which gives the specific activity of different fractions, expressed arbitrarily as the ratio of counts/min./ml. to optical density. The specific activity increased steadily from tubes 22 to 28, but then fell again,

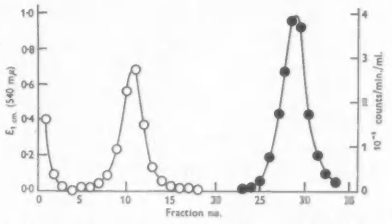

Fig. 3. Separation of 20 mg. of haemoglobin (○) from 30 mg. of human [^{131}I]albumin (●) after 40 hr. electrophoresis in a sucrose gradient containing 0·025 M-borax (pH 9·2). Current 23–17 mA. The haemoglobin was located by absorption at 540 mμ, and the albumin by its radioactivity.

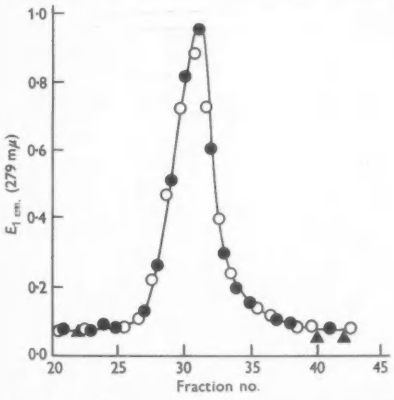

Fig. 2. Ultraviolet-absorption curve obtained after electrophoresis of 30 mg. of human albumin for 45 hr. in a sucrose gradient containing 0·025 M-borax (pH 9·2). Current 23–17 mA. Fractions collected manually are shown by filled circles, those collected automatically in a duplicate run by open circles. Triangles denote absorption arising from the batch of sucrose used.

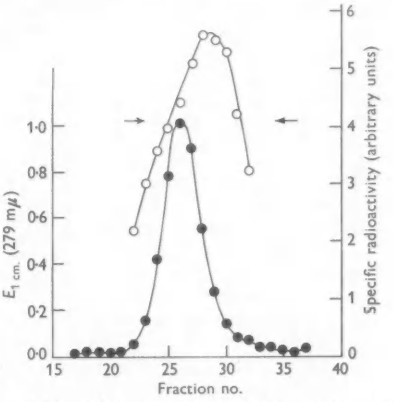

Fig. 4. Ultraviolet-absorption curve (●) obtained after electrophoresis for 40 hr. of 45 mg. of human [^{131}I]-albumin, in a sucrose gradient containing 0·025 M-borax (pH 9·2). Current 22–16 mA. Specific radioactivities of the fractions (○) and of the starting material (arrows) are also shown.

behaviour which is referred to in the Discussion. When the contents of tube 26 were mixed with a small volume of concentrated untreated albumin and re-run, the curves obtained were still very similar. In a repetition of this experiment, at the conclusion of the run the contents of the gradient column were expressed through a capillary tube at the top of the column. The last centimetre of this tube, which extended approximately 3 cm. outside the gradient tube, was curved downwards to facilitate collection of fractions while minimizing mixing. The curves obtained were still of the same type as before.

Curves obtained after 1- and 2-day runs on insulin in ammonium acetate buffer and ethanol are shown in Fig. 5. The main feature to note is the shoulder on the trailing edge of the peak. The spread was somewhat greater than in an experiment with insulin (not shown) in the sucrose gradient.

Electrophoresis of the slow-reacting substance A in a sucrose gradient was carried out for 24 hr., after which the 3·0 ml. fractions obtained from the column were tested biologically by Mr Brocklehurst with the results shown in Fig. 6. More than 70% of the activity applied to the column was accounted for in tubes 32–37. As it was not possible to separate the active material from the sucrose by dialysis, experiments were carried out in the volatile system used for insulin, but at a lower pH (8·0). A typical set of results is illustrated in Fig. 7, which shows that a reasonably sharp peak is obtainable under these conditions. A minor distribution of active material, which differed qualitatively in biological behaviour from the major component, appeared moving more slowly than the main peak. There is reason to believe that the minor component may be a breakdown product of the other.

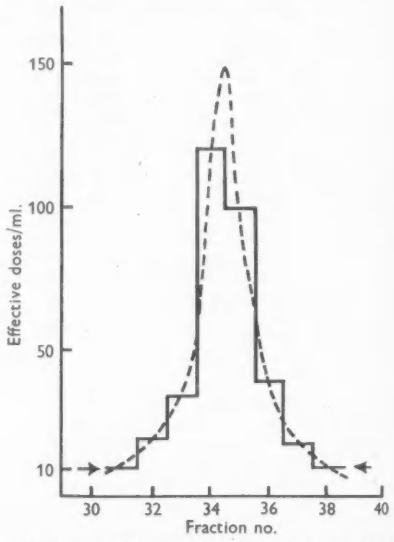

Fig. 6. Histogram showing biological activity of fractions obtained after electrophoresis of a sample of slow-reacting substance A for 22 hr. in a sucrose gradient containing phosphate buffer (pH 8·0, 0·05 *I*). Current 24–20 mA. Arrows show limit of sensitivity of biological assay.

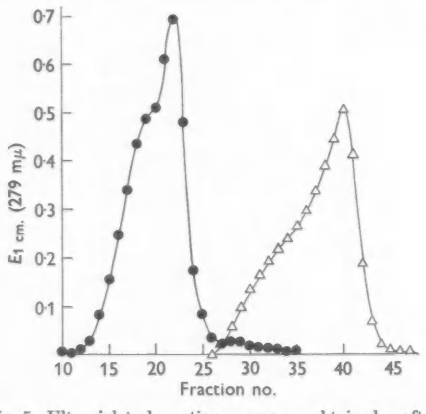

Fig. 5. Ultraviolet-absorption curves obtained after electrophoresis of 50 mg. of insulin in an ethanol gradient containing 0·025 M-ammonium acetate adjusted to pH 9·0 with ammonia: (●) after 16 hr. (current 21–20 mA); (△) after 44 hr. (current 16–14 mA).

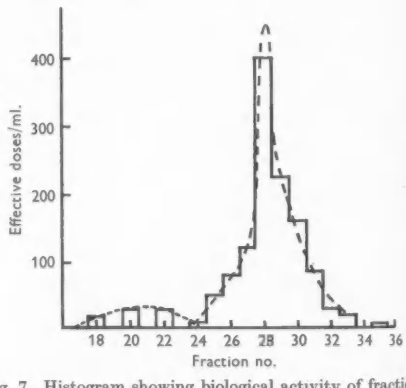

Fig. 7. Histogram showing biological activity of fractions obtained after electrophoresis of a sample of slow-reacting substance A for 17·5 hr. in an ethanol gradient containing 0·025 M-ammonium acetate adjusted to pH 8·0 with ammonia. Fractions from minor peak showed a modified gut response (see text).

DISCUSSION

Some of the advantages of this type of electrophoresis have already been mentioned in the introduction. It seems probable, however, that the usefulness of the method will depend largely both on the reproducibility of the separations which can be obtained and on the shapes of bands after separation, with particular reference to degree of resolution. The results recently published by Berg & Beeler (1958) show an apparently good resolution for human serum and plasma, although, as they themselves state, the method of protein estimation used must have led to an exaggeration of the albumin peaks. As appears in Fig. 2, very precise reproducibility from experiment to experiment has been obtained with human serum albumin. The shape of the band of human albumin after electrophoresis has been compared with that calculated for diffusion alone after the same time. Although the latter, as must be expected, is considerably sharper, the greater spread of the former is within acceptable limits, as 84% of the albumin in each experiment (Fig. 2) is accounted for in five tubes. A slight asymmetry, consisting mainly of a more diffuse leading edge, is, however, detectable. Since the albumin used was of known electrophoretic homogeneity (Charlwood, 1954), this phenomenon requires explanation. Several possibilities have been considered, including 'tailing', and viscosity gradient in the tube. It seemed likely that, if the gradient were allowed to run out too fast, 'tailing' would result from the increased drag experienced by regions of the very viscous sucrose solution nearer to the walls of the main tube. In the experiment with the apparatus specially modified to permit running out from a capillary fixed at the top of the gradient, the usual band shape was found. Since reversal of the shape of the band did not take place, it appears that tailing cannot be of more than very minor importance at the rates of running out used in the present work. It seems likely that effects of this kind will be even less important when the gradient has been made from ethanol instead of from sucrose, because of the much lower viscosities of solutions of the former substance. In the present work both ethanol and sucrose gradients have been run out at approximately 1·5 ml./min. This is considerably slower than the rate suggested for ethanol gradients by Svensson & Valmet (1955). The marked asymmetry of certain components in the diagrams of human serum (Svensson *et al.* 1957) may perhaps have resulted from unduly fast running out, but unfortunately these authors did not state the rate at which their sucrose gradients were run out.

When trace-iodinated human serum albumin was used, the peak of radioactivity moved slightly further up the tube than did the peak of optical density. A leading edge showing higher radioactivity than that of the main band has previously been reported by Gabrieli, Goulian, Kinersly & Collet (1954), who subjected commercially iodinated (Abbott) human serum albumin to electrophoresis on paper. On the other hand, trace-iodinated albumins (Cohen & Shamroth, 1958) do not show this effect by paper electrophoresis. The specific activities of the albumin fractions (Fig. 4) showed a maximum, instead of rising steadily as might be expected if, on the average, the iodinated molecules had a slightly higher mobility than those without iodine. This behaviour was reproduced with the albumin from the Porath column and was not therefore something peculiar to the other batch. Further, in the experiment mentioned above, in which fractions were taken out from the top of the tube, the curve of specific activity again showed a maximum and, indeed, was very similar to that obtained by running out from the bottom. This effect, which is capable of several interpretations, apparently does not derive from the conditions of running out, but must be related to the relative rates of movement of the protein species present. Evidently the specific activity measurements provide a refined indication of very slight degrees of separation.

The main purpose of the experiments with insulin was to ascertain whether a substance of large molecular weight could be made to move reproducibly and without excessive spreading in an ethanol gradient. The degree to which this has been achieved is shown in Fig. 5, which gives the band shapes obtained after 16 hr. and in a second experiment under the same conditions except that it extended to 44 hr. Rather similar unsymmetrical patterns have been reported for this protein by Timasheff, Brown & Kirkwood (1953), who used barbiturate buffer at pH 8·6 in the Tiselius apparatus. On the other hand, the existence of a boundary of lower mobility could not be confirmed in the present work when insulin was examined at pH 9·2 in a sucrose gradient. Possibly the boundary shape first noted by Timasheff *et al.* (1953) is due to an association–dissociation reaction which is suppressed in concentrated sucrose solutions.

The experiments with albumin–haemoglobin mixtures (see Fig. 3), and other evidence in this work, indicate that electrophoresis in a density gradient is capable of providing good resolution with moderate loads of protein. Satisfactory runs have been made with 250 mg., but there must be a limit to the quantities which can be separated by this method; for instance, it could hardly be expected to operate on the scale which can be used for zone electrophoresis in a solid matrix (Porath & Gedin, 1957). On the other hand, the absence of a

matrix has certain advantages. Since adsorption is ruled out, choice of the inert material in the column becomes much simpler. The requirements for the material from which the gradient is formed are that it should be a non-electrolyte, unable to react with the substances being investigated and, in preparative work, easily separable from these substances. In the present work only sucrose and ethanol have been used for the gradients, but for other problems different materials might well be more suitable. The outstanding advantage of the gradient method, due to the relatively small surface area of solid exposed to the samples, has been illustrated in the present work by the experiments with slow-reacting substance A. Thus, whereas the ease with which this material is adsorbed has prevented useful electrophoresis in other zone systems, adequately sharp bands have now been obtained both in sucrose and in ethanol gradients. Thus it is probable that this type of zone electrophoresis will be of increasing usefulness in the separation of biologically active substances which are readily adsorbed on the matrix materials used for zone electrophoresis.

SUMMARY

1. A density-gradient electrophoresis apparatus somewhat modified from that designed by Svensson is described. Experiments have been carried out in gradients made from ethanol and from sucrose.

2. The reproducibility of movement and shape of bands of human albumin and the completeness of separation of this protein from a band of haemoglobin have been studied in the sucrose gradient.

3. The specific activities of the fractions thus obtained from human plasma albumin trace-iodinated with ^{131}I have been found to vary considerably and to show a maximum just preceding the maximum protein concentration as measured by ultraviolet-light absorption.

4. Insulin has been caused to migrate in both the sucrose and the ethanol gradients; in the latter the protein concentration curve possessed a shoulder.

5. Suitable conditions have been found for the electrophoresis of slow-reacting substance A, a substance causing constriction of the guinea-pig ileum.

We are grateful to Dr A. S. McFarlane and Dr S. Cohen for numerous discussions and for preparation of some of the protein samples.

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Assimilation of Carbon Dioxide by Yeasts

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The formation of carbon dioxide during glucose oxidation or fermentation by yeasts is well established, The fixation of carbon dioxide is less well known, though its existence is supported by the following evidence: (1) Carbon dioxide is necessary for the growth of yeasts (Rockwell & Highberger, 1927) and the synthesis of succinic acid by *Saccharomyces cerevisiae* (baker's yeast) (Kleinzeller, 1941). (2) Both carbon dioxide and dicarboxylic acids promote substrate oxidation by baker's yeast (Sperber & Runnström, 1939; Stoppani, 1951) but their actions are not additive. The replacement effect indicates that carbon dioxide is incorporated into dicarboxylic acids, which are the true stimulants of substrate oxidation. (3) During pyruvate oxidation in a medium

containing bicarbonate, baker's yeast fixes carbon dioxide (Runnström & Sperber, 1942). (4) Baker's yeast incorporates $^{11}CO_2$ (Ruben & Kamen, 1940) and $^{14}CO_2$ during glucose oxidation (Liener & Buchanan, 1951). (5) After pyruvate oxidation in the presence of $^{14}CO_2$, baker's-yeast amino acids contain radiocarbon and the labelling pattern indicates that the first fixation product is a C_4 intermediate (Davis, Cheldelin, Christensen & Wang, 1956).

Carbon dioxide is incorporated into cell metabolism by several reactions (cf. Utter & Wood, 1951). The condensation with a C_3 compound is one of the most important and leads to dicarboxylic acids which, through the citric acid cycle, promote cell respiration and liberation of energy (Wiame, 1957) or contribute to the synthesis of amino acids and other essential cell components (Roberts, Cowie, Abelson, Bolton & Britten, 1955; Wiame, 1957). Reactions of the citric acid cycle can be shown to take place in *S. cerevisiae*, either with living cells or cell-free preparations (cf. Wiame, 1957), and many workers claim that the cycle operates as in animal tissues (Martius & Lynen, 1950; Weinhouse & Millington, 1947; Barron, Ardao & Hearon, 1950; etc.). However, important effects dependent on the cycle operation cannot be demonstrated with baker's yeast (Krebs, Gurin & Eggleston, 1952), and on these grounds it is still a subject of controversy (Krebs, 1954) whether in this organism the cycle is the main pathway of substrate oxidation and energy liberation.

The operation of the citric acid cycle implies a flow of carbon through definite positions of the cycle intermediates and further, in aspartic and glutamic acid, two amino acids closely related to the cycle (Roberts *et al.* 1955). When $^{14}CO_2$ is used, the fixation reactions yield dicarboxylic acids (malic or oxaloacetic acid) labelled in C-4 and, accordingly, the aspartic acid formed by transamination of oxaloacetate would be expected to be labelled similarly in C-4. Also the position of ^{14}C in glutamic acid can be predicted as, on passing around the cycle, C-4 of oxaloacetic acid labels α-oxoglutaric (and glutamic) acid in C-1. Finally succinic acid should not contain ^{14}C if the cycle operates in one direction only, but partial equilibration of oxaloacetic with malic, fumaric and succinic acid, or the reverse operation of the cycle (or some of its reactions), may yield succinic acid symmetrically labelled in the carboxyl groups.

The experiments reported below have been planned in order, to establish (i) the mechanism of carbon dioxide fixation by *S. cerevisiae*, (ii) its metabolic role, and (iii) to test the validity of isotope-distribution models based upon the cycle operation in living yeast cells under different environmental conditions. Some complementary experiments have also been carried out with *Saccharomyces carlsbergensis*.

A preliminary account of this work has already appeared (Stoppani, de Favelukes, Conches & Sacerdote, 1957).

EXPERIMENTAL METHODS

Organisms and medium. Commercial preparations of baker's yeast were used in most of the experiments. Their bacteriological purity was checked and found satisfactory. Similar results were obtained with a pure strain of *S. cerevisiae* grown at 22° in a medium containing: malt extract (commercial preparation), 200 g.; Difco yeast extract, 3 g.; KH_2PO_4, 2 g.; $(NH_4)_2SO_4$, 0·5 g.; urea, 1 g. and glucose, 10 g., in 1 l. of tap water, under continuous aeration. The final pH of the medium was about 6·0. *S. carlsbergensis* was cultured from a pure strain in the same medium but without aeration. The yeast cells were washed with water in the centrifuge, suspended in water and starved by aeration at 22° for at least 12 hr. before use. All the operations were carried out under sterile conditions. The final concentration of the yeast suspensions was estimated in a photoelectric colorimeter by comparison with a standard suspension, the dry weight (100°) of which was established.

Enzymes. Carboxylase was prepared according to Green, Herbert & Subrahmanyan (1941) and yeast transaminase, as described by Bigger-Gehring (1955), from yeast acetone-dried powder; 50 mg. of acetone-dried powder was rubbed up with 0·1 M-phosphate buffer (pH 7·8; 1 ml.) and the extract dialysed against 0·02 M-KCl at 1–2° for 6 hr. The suspension was centrifuged and the clear supernatant tested for transaminase activity. Heart-muscle transaminase was the precipitate obtained at pH 4·2 from Cohen's (1940) transaminase preparation, but the grinding up of pig-heart muscle mince was replaced by homogenizing with the Waring Blendor.

Radioactive bicarbonate. This was obtained from The Radiochemical Centre, Amersham, Bucks. The specific activity was 1 mc/0·17 m-mole which under the conditions of radioassay used was equivalent to $1·18 \times 10^8$ counts/min. The radioactive bicarbonate was diluted to 5·8 ml. with water (CO_2-free).

Chemicals. Analytical-grade reagents were used throughout. Pyruvic acid was obtained by continuous ether extraction at pH 1 from sodium pyruvate prepared according to Robertson (1942). Acetaldehyde was distilled from a commercial preparation, dissolved in water and stored at 4°. The strength of the solutions was checked when necessary by the bisulphite method (Elliott, Benoy & Barker, 1935). Antimycin A was obtained from the Wisconsin Alumni Research Foundation (P.O. Box 2059, Madison 1, Wis., U.S.A.).

Manometric methods. Oxygen uptake and production of CO_2 were measured in the Warburg apparatus at the temperature stated in each case. Carbon dioxide was determined by the direct method. Each flask contained a total volume of 2 ml. of fluid.

Incubation techniques. In order to study the kinetics of $^{14}CO_2$ fixation, a closed system (cf. Stoppani, Fuller & Calvin, 1955) was used. The reaction chamber was a 125 ml. separating funnel with the drain tube cut obliquely at 5 mm. below the stopcock. The top orifice was closed by a

rubber bung pierced with a glass tube connected to a 10 ml. graduated glass syringe (volume compensator) lubricated with paraffin oil. Four reaction vessels were fixed on a shaking machine (used for Kahn tests) with the reaction chamber vertically and the volume compensator horizontally placed on the carriage. The substrate and radioactive bicarbonate were mixed successively with the yeast suspension in the reaction vessel. This was immediately closed with the stopper attached to the volume compensator, the plunger of which had been adjusted previously to the 10 ml. mark. Shaking was started and, after the required time, samples (about 1 ml.) of yeast suspension were taken by opening the stopcock of the separating funnel and pushing the plunger of the volume compensator to the corresponding mark. In this way a closed atmosphere was secured throughout the experiment, any losses of radioactive bicarbonate at the moment of taking the yeast samples being avoided. These precautions were necessary as, in most of the experiments, the initial pH of the yeast suspension was sufficiently acid to transform all the bicarbonate into CO_2.

For experiments with pure O_2 or N_2 in the gas phase, each reaction vessel was provided with a flushing system; the gas escaped through a steel needle inserted through the bung. The reagents were quickly added through the needle with a thinner needle attached to a calibrated syringe; in this manner almost air-tight conditions were maintained during the additions. The rest of the procedure was as described in the experiments with air.

The pH values of the incubation mixtures were measured with a glass electrode before the addition of the radioactive bicarbonate; the addition of the bicarbonate to the media of low pH raised the pH by about 0·3–0·4 when acetic acid or pyruvic acid was present and about 1·0 when phosphate buffer was the only electrolyte present. The values given are approximately the ones obtained after the addition of the bicarbonate. At an initial pH above 7·0 the influence of bicarbonate addition was far less important, as the increase was about 0·1–0·2. No significant variation in the yeast metabolism can be expected from differences of external pH in the ranges mentioned as the intracellular pH is largely independent of the medium pH (Krebs *et al.* 1952).

Analytical methods. The yeast samples were added to and thoroughly mixed with 9 ml. of methanol previously weighed in a tared 15 ml. centrifuge tube. The tubes were weighed, spun at 2500 rev./min. and the precipitate was discarded. The supernatants were placed in a vacuum system provided with solid CO_2–ethanol and liquid-air traps, and evaporated at low temperature to 0·4–0·8 ml. volume. The residues were chromatographed with the phenol–water and butanol–propionic acid–water solvent systems described by Benson *et al.* (1950). The chromatograms were radioautographed on X-ray film for 2–3 weeks and the radioactive compounds identified by cochromatography and radioautography with pure specimens.

Glutamic and aspartic acid were determined, after elution from the chromatograms, by the methods of Russell (1944) or Moore & Stein (1948). Eluates from non-radioactive areas were used as controls. Pyruvic acid was estimated according to Friedemann & Haugen (1943).

Preparation of radioactive samples. In order to measure the total ^{14}C fixed by the yeast cells, 300 μl. of the methanolic yeast suspension was evenly distributed on 3·8 cm.^{2} aluminium cups, evaporated to dryness on a turning table under a stream of hot air (Benson, 1949) and counted. When the pH of the incubation medium was high, the methanolic suspensions were acidified in order to remove the labelled CO_2. The thickness of the samples was always less than 0·5 mg./cm.^{2} and corrections for self-absorption were therefore unnecessary. After centrifuging the yeast suspension, 300 μl. of the clear supernatant was plated as above and counted. The activity represented the ^{14}C fixed in the methanol–water-soluble substances. The difference between this and the activity found in the total extracts corresponds to ^{14}C fixed in proteins and other materials insoluble in the methanol–water mixture.

The $BaCO_3$ obtained from total or partial degradation of radioactive compounds was filtered off on tared filter paper (Schleicher and Schull 589), washed twice with 10 ml. of CO_2-free water and 5 ml. of acetone, and dried under a stream of hot air, and placed on the aluminium cups and reweighed in order to calculate the weight of $BaCO_3$.

Degradation of radioactive compounds. Aspartic, glutamic and succinic acid were degraded in order to establish the intramolecular distribution of ^{14}C. The radioactive compounds eluted from the chromatograms, conveniently diluted with the respective carrier, were subjected to combustion with the mixture of Van Slyke, Plazin & Weisiger (1951) in an all-glass apparatus, based on the Lindenbaum, Schubert & Armstrong (1948) model. Six sets of apparatus were installed on a vacuum line provided with a 100–1 mm. Hg vacuum gauge. These modifications enabled the combustion train to be operated smoothly and the results were much more reproducible than with the original device. The CO_2 was precipitated as $BaCO_3$; this was washed twice with CO_2-free water, and filtered and mounted as described above. The C-1 of glutamic acid and the C-1 and C-4 of aspartic acid were quantitatively liberated at pH 2·5 with chloramine-T (Van Slyke, Dillon, MacFadyen & Hamilton, 1941). The operation was performed in Warburg manometers with 0·2 ml. of 5N-NaOH (carbonate-free) in the centre well (without filter paper). After all the CO_2 was fixed, the carbonate was transferred quantitatively to centrifuge tubes with 2 ml. of water, and 60 μmoles of carbonate carrier added and the carbonate precipitated as $BaCO_3$. The $BaCO_3$ was washed twice in the centrifuge with water, filtered off and mounted on paper disks. C-4 of aspartic acid was liberated by transamination of aspartate with α-oxoglutarate and decarboxylation of oxaloacetate with aniline at pH 5·0, a heart-muscle transaminase preparation (Krebs, 1950) being used. The CO_2 evolved was fixed in 0·2 ml. of 5N-NaOH placed in the centre well of the Warburg manometer and precipitated as $BaCO_3$ as in the decarboxylation with chloramine-T. C-5 of glutamic acid and the carboxyl carbons of succinic acid were liberated by the Schmidt reaction (Phares & Long, 1955).

After elution from the chromatograms, portions of the radioactive amino acids were diluted with carrier and subjected to degradation. The yield of CO_2 liberated was determined either by a separate manometric measurement (decarboxylation with chloramine-T or the transaminase–aniline system) or by the weight of the $BaCO_3$ (total combustion and Schmidt reaction), and corrections for losses were introduced when required. As the amount of carrier varied according to the degradation procedure, the activities obtained are not referred to a weight unit; this is un-

necessary because of the separate control of each degradation procedure. With aspartic acid, the activity (a) in C-4 was obtained from the CO_2 liberated with the transaminase–aniline system; the activity in C-1 by subtracting a from the activity in the CO_2 liberated with chloramine-T (b) and the ^{14}C in the amino and methylene carbons by subtracting b from the activity (c) in the CO_2 obtained after the total combustion. The percentage proportion of a is established in relation to c. With glutamic acid a similar system of calculation was used.

The specific activity of ^{14}C in the carboxyl groups of succinic acid was obtained directly from the CO_2 liberated with the Schmidt reaction and the specific activity in the methylene carbons by difference between the activity in the carboxyl groups and the total activity. Specific activities are expressed as counts/min./mg. of $BaCO_3$.

Assay of radioactive samples. Activity measurements were carried out with a Geiger–Müller end-window counter connected to a decimal scaler. The activity values were corrected for background, self-absorption and dead time of the counting system and are expressed as counts/min. at infinite thinness. All measurements were standardized with reference to a standard of ^{14}C to which 700 counts/min. was ascribed. Radioactive compounds were also counted directly on the chromatograms. The active areas were located with the aid of the radioautograph and counted with a Scott-type Geiger–Müller tube placed directly on the paper. The neighbouring radioactive areas were covered with masks. Activity values were corrected for background and dead time of the counting system and standardized as above. The number of counts recorded was enough to make the standard error less than 3%.

Expression of results. The ^{14}C fixed by the yeast cells (R), expressed as counts/min./mg. of cells (dry wt.), is calculated with equation (1), where 700 is the activity ascribed to the ^{14}C standard;

$$R = 700 \times C_y \times W_m \times V/(0{\cdot}81 \times v \times C_{st} \times W_y \times Y) \quad (1)$$

[C_y, counts/min. of yeast sample; W_m, the weight of the suspension of yeast cells in the 90% (v/v) methanol–water mixture; V, volume of the incubation mixture; 0·81, sp.gr. of the 90% (v/v) methanol–water mixture; v, volume of yeast suspension taken for the assay of radioactivity; C_{st}, activity found with the ^{14}C standard; W_y, weight of the sample of incubation mixture; Y, amount (mg.) of yeast used in the experiment]. It is assumed that the sp.gr. of the incubation mixture is 1·0 and that small variations in the volume of the sample of yeast suspension do not affect significantly the sp.gr. of the 90% (v/v) methanol–water mixture. The ^{14}C fixed in the methanol–water-soluble compounds is calculated in a similar way.

When the radioactivity was counted directly on the chromatograms the relative activity of each substance was calculated with the equation $A_X(\%) = (C_X/C_N) \times 100$, where C_X was the number of counts of substance X and C_N the total activity counted on the chromatogram. The ^{14}C fixed in X by 1 mg. of cells was obtained by multiplying A_X by the total ^{14}C fixed by 1 mg. of cells in the methanol–water-soluble substances.

RESULTS

Fixation of carbon dioxide by Saccharomyces cerevisiae *with acetate or acetaldehyde as substrates*

Fixation and distribution of $^{14}CO_2$. Baker's yeast incorporates carbon dioxide during acetate and acetaldehyde oxidation. The ^{14}C fixed depends on the amount of substrate oxidized (Table 1), which shows that besides possible exchange reactions the $^{14}CO_2$ incorporation is bound to metabolic processes. With acetate (Fig. 1) at the beginning of the experiment the oxygen uptake and carbon dioxide fixation are relatively slow, which corresponds to the 'induction period' of acetate oxidation

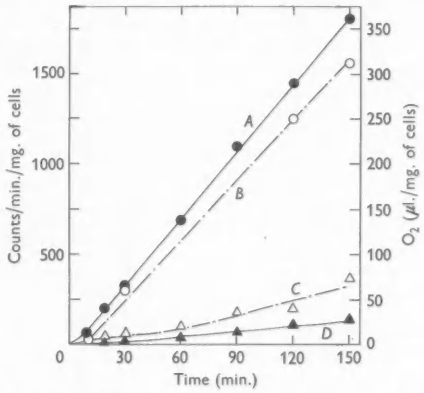

Fig. 1. Fixation of $^{14}CO_2$ and oxygen uptake of *S. cerevisiae* with acetate as substrate. Yeast, 21 mg./ml.; 20 mM-acetic acid; 0·4 M-phosphate; pH 2·8 in the manometric experiment, 3·1 in the fixation experiment. In the fixation experiment 4·2 μmoles of $NaH^{14}CO_3$ ($3{\cdot}1 \times 10^6$ counts/min.). Total vol.: 2 ml. in the manometric experiment and 10 ml. in the fixation experiment. Temp. 30°. Air was in the gas space. *A*, Oxygen uptake with acetate and *D*, control. *B*, $^{14}CO_2$ fixation with acetate and *C*, control.

Table 1. *Oxygen uptake and* $^{14}CO_2$ *fixation by* Saccharomyces cerevisiae *with acetate and acetaldehyde as substrates*

Experimental conditions as in	Substrate	Q_{O_2}	^{14}C fixed (counts/min./mg. of cells/hr.)
Fig. 1	Acetate	13·8	870
Fig. 1	None	1·1	103
Fig. 2	Acetaldehyde	15·3	900
Fig. 2	None	2·9	90

(Wieland, Probst & Crawford, 1938). With acetaldehyde (Fig. 2) the initial oxygen uptake is faster than the carbon dioxide fixation, which shows the same delay as with acetate. This difference between the rate of oxidation and $^{14}CO_2$ fixation can be attributed to the oxidation of acetaldehyde to acetic acid, which apparently does not involve carbon dioxide fixation. In order to increase the permeability of yeast to acetate, with this substrate the initial pH of the medium was decreased. The strong acidity did not affect qualitatively the fixation reactions as, with acetaldehyde, experiments carried out at low and high pH gave, after sufficient time of incubation, the same isotope distribution (Table 2).

The substances labelled after $^{14}CO_2$ fixation are listed in Table 2. All are soluble in the methanol–water phase because the lack of nitrogen compounds and growth factors in the medium prevented the synthesis of yeast proteins. The radioactive compounds may be grouped as (1) Krebs-cycle intermediates, (2) amino acids, and (3) glycolysis intermediates. The largest reservoirs of ^{14}C are aspartic and glutamic acid and, among the Krebs-cycle intermediates, malic and the tricarboxylic acids (citric, aconitic and *iso*citric acid). Most of the amino acids labelled are related to the Krebs-cycle intermediates. With acetaldehyde as substrate, succinic, fumaric, phosphoglyceric and phosphopyruvic acids fix ^{14}C, which leads to the assumption that the reducing power of acetaldehyde reverses some reactions of the Embden–Meyerhof sequence, and Krebs cycle. A similar but more evident effect will be described with glucose.

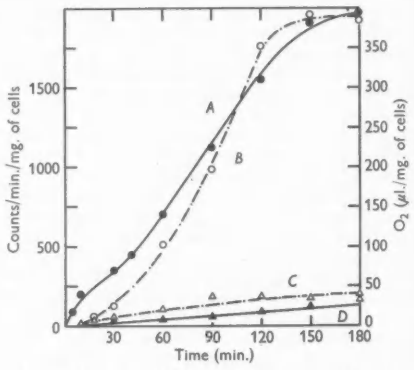

Fig. 2. Fixation of $^{14}CO_2$ and oxygen uptake by *S. cerevisiae* with acetaldehyde as substrate. 20 mM-Acetaldehyde; 0·4 mM-phosphate; pH 2·8 in the manometric experiment, 3·1 in the fixation experiment. Other experimental details are as in Fig. 1. *A*, Oxygen uptake with acetaldehyde and *D*, control. *B*, $^{14}CO_2$ fixation with acetaldehyde and *C*, control.

Table 2. *Distribution* (%) *of* $^{14}CO_2$ *fixed by* Saccharomyces cerevisiae *with acetaldehyde or acetate as substrate*

The reaction mixture contained 210 mg. of yeast cells, 20 mM-acetaldehyde (or acetate), 0·4 mM-phosphate and 4·2 µ-moles of $NaH^{14}CO_3$ ($3·1 \times 10^6$ counts/min.). Incubation was for 120 min. with acetaldehyde and 150 min. with acetate. Temp. 30°. Air was in the gas space. Total ^{14}C fixed (counts/min./mg. of cells): 1510 (with acetaldehyde, pH 3·1), 1700 (with acetaldehyde, pH 7·0) and 1505 (with acetate, pH 3·1). ^{14}C fixed in the methanol–water-soluble substances: 1600 (with acetaldehyde, pH 3·8), 1520 (with acetaldehyde, pH 7·0) and 1400 (with acetate).

	^{14}C fixed (% of total in the methanol–water-soluble substances)		
Substance	Substrate: acetaldehyde (pH 3·8)	Substrate: acetaldehyde (pH 7·0)	Substrate: acetate (pH 3·1)
Krebs-cycle intermediates			
Malic acid	7·5	7·3	5·6
Citric, aconitic and *iso*citric acid	3·7	2·7	3·8
Fumaric acid	1·1	0·9	0
Succinic acid	1·0	1·2	0
Amino acids			
Aspartic acid	58·0	60·2	59·6
Glutamic acid	17·5	17·8	21·9
Alanine	2·3	2·5	2·0
Threonine	0	0	1·7
Glycine	2·5	2·5	1·5
Arginine–ornithine	0·9	0·9	0·8
Glycolysis intermediates			
Phosphoglyceric acid	0·9	0	0
Phosphopyruvic acid	0·9	0·6	0
Other substances	3·7	3·4	3·1

The oxidation of the yeast endogenous substrates brings about carbon dioxide fixation, which is differentiated from the fixation associated with substrate oxidation by the far smaller amounts of ^{14}C incorporated (cf. Figs. 1 and 2) and of compounds labelled (Table 3). Furthermore, aspartic and glutamic acids fix relatively more ^{14}C than during acetate or acetaldehyde oxidation. The distribution pattern varies slightly from one yeast preparation to another and, in some, the tricarboxylic acids, fumaric acid and succinic acid also appear radioactive.

The kinetics of $^{14}CO_2$ fixation with acetate as substrate (similar results were obtained with acetaldehyde) are shown in Fig. 3, where only those compounds presumably related to the fixation reactions (aspartic, malic, glutamic and the tricarboxylic acids) have been plotted. According to the time of incubation, three different distributions of ^{14}C can be described. In the first 30 min. aspartic acid has most of the activity, which decreases continuously. Also the curve of malate shows negative slope, but this may not be significant as the sign of the slope depends upon a single experimental point at a time when the total counts in the cell are very low and the error involved in the determinations is consequently higher. On the other hand, the incorporation of ^{14}C in glutamic acid increases continuously, and after 30 min. nearly reaches its maximum. In the 30–120 min. interval, aspartic acid shows a minimum whereas glutamic, malic and the tricarboxylic acids have maxima. From 120 min. onwards the relative distribution of ^{14}C in aspartic acid increases steadily, whereas decreases occur in glutamic, malic and the citric acid group. If the absolute radioactivity fixed by each compound is plotted against time of incubation (Fig. 4) a close relationship with the percentage-distribution plot is observed and it becomes evident that by the end of the experiment aspartic acid is the largest reservoir of ^{14}C.

The kinetics of $^{14}CO_2$ fixation during the oxidation of endogenous substrates have also been studied. In the percentage-distribution plot (Fig. 5), only aspartic acid has a negative slope and after incubation for 30 min. a steady-state distribution appears. The curves do not overlap, which means a striking and systematic difference from the plots of ^{14}C distribution after substrate oxidation. Accordingly, the absolute distribution of ^{14}C in each compound increases continuously throughout the incubation (Fig. 6).

Table 3. *Distribution* (%) *of* $^{14}CO_2$ *fixed by* Saccharomyces cerevisiae *during the oxidation of endogenous substrates*

Experimental conditions were as described in Table 2. Substrate was omitted. Incubation was for 2 hr. Total ^{14}C fixed (counts/min./mg. of cells): 178; ^{14}C fixed in the methanol–water-soluble substances: 161.

Substance	^{14}C fixed (% of total in the methanol–water-soluble substances)
Krebs-cycle intermediates	
Malic acid	3·6
Amino acids	
Aspartic acid	65·4
Glutamic acid	25·6
Alanine	2·2
Glycine	1·8
Other substances	1·4

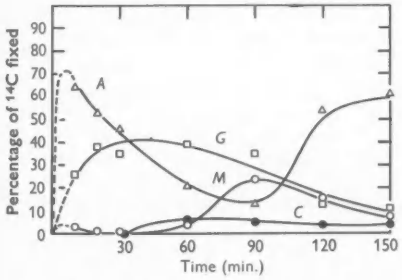

Fig. 3. Kinetics of ^{14}C distribution after $^{14}CO_2$ fixation by *S. cerevisiae* with acetate as substrate. Experimental conditions were the same as in Fig. 1 (fixation experiment). *A*, Aspartic acid; *G*, glutamic acid; *M*, malic acid; *C*, citric, aconitic and *iso*citric acid.

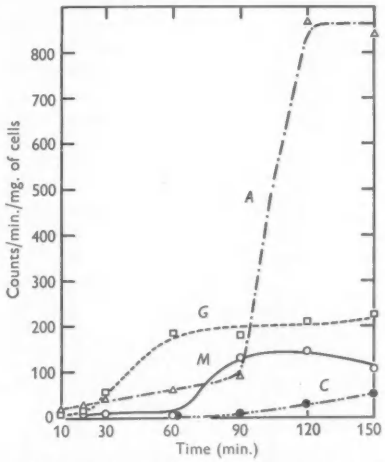

Fig. 4. Absolute distribution of $^{14}CO_2$ fixed by *S. cerevisiae* during acetate oxidation. Details as for Fig. 3.

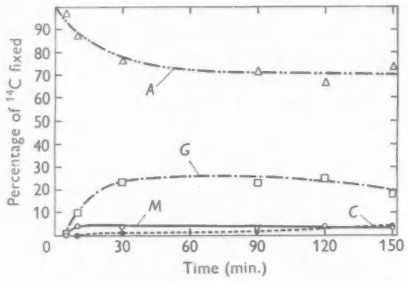

Fig. 5. Kinetics of ^{14}C distribution after $^{14}CO_2$ fixation by *S. cerevisiae* during the oxidation of endogenous substrates. Experimental conditions were the same as in Fig. 2. Acetaldehyde was omitted. *A*, Aspartic acid; *G*, glutamic acid; *M*, malic acid; *C*, citric, aconitic and *iso*citric acid.

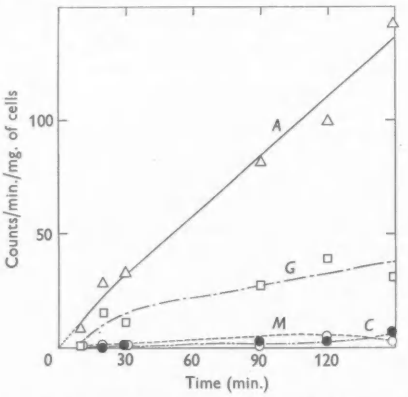

Fig. 6. Absolute distribution of $^{14}CO_2$ fixed by *S. cerevisiae* during the oxidation of endogenous substrates. Details as in Fig. 5.

Fixation of ^{14}C *in aspartic and glutamic acid.* For the understanding of the kinetic experiments summarized above, the specific activity and intramolecular distribution of ^{14}C in aspartic and glutamic acid during the incubation are essential. Both acids are in equilibrium with oxaloacetic and α-oxoglutaric acid, two significant intermediates of carbon dioxide assimilation, and the respective transaminations have been shown in *Saccharomyces fragilis* (Bigger-Gehring, 1955) and confirmed in *S. cerevisiae* (Table 4).

Aspartic and glutamic acid were contained free in the respective proportions of about 3 and 11 μmoles/100 mg. of dry cells of the yeast preparations used (Table 5), which is consistent with previous observations of Halvorson & Spiegelman (1953) and other workers. The pool of these amino

Table 4. *Oxaloacetate–glutamate transaminase in cell-free extracts of* Saccharomyces cerevisiae

The reaction mixture (2 ml.) contained 0·6 ml. of dialysed yeast extract (prepared as described under Methods), 0·1 M-phosphate buffer, pH 7·8, and 0·01 M-amino acid or -keto acid or both. Incubation was for 2 hr. at 30°. The reaction was stopped by addition of 10 ml. of absolute ethanol and boiling for 2 min. in a water bath. The precipitate was discarded after centrifuging and the supernatant evaporated to 2 ml. Amino acids were separated on paper by chromatography with the phenol–water-solvent system and, after elution, estimated by the Moore & Stein (1948) method.

Additions	Amino acid formed (μmoles) Glutamate	Aspartate
None	0	0
L-Glutamate	—	0
L-Aspartate	0	—
α-Oxoglutarate	0·4	—
Oxaloacetate	—	3·4
α-Oxoglutarate + L-aspartate	6·9	—
Oxaloacetate + L-glutamate	—	9·6

Table 5. *Concentration and specific activity of free aspartic and glutamic acids in* Saccharomyces cerevisiae *during* $^{14}CO_2$ *fixation with acetaldehyde as substrate*

Amounts of amino acid and radioactivity were measured in samples of aspartic and glutamic acid eluted from the chromatograms of the experiment on acetaldehyde oxidation (at pH 7·0) summarized in Table 2. Amino acids were measured according to Russell (1944).

Time of acetaldehyde oxidation (min.)	Aspartic acid (μmoles/100 mg. of cells)	Aspartic acid Specific activity (counts/min./μmole)	Glutamic acid (μmoles/100 mg. of cells)	Glutamic acid Specific activity (counts/min./μmole)
10	3·0	210	11·0	13
20	2·0	1180	16·0	83
30	3·0	1300	—	—
60	—	—	42·0	236
90	4·0	1440	—	—
120	18·0	2800	42·0	272
150	—	—	44·0	332
180	25·0	2400	37·0	1160

Table 6. *Distribution of* ^{14}C *in aspartic acid from* Saccharomyces cerevisiae *after fixation of* $^{14}CO_2$ *with acetaldehyde and acetate as substrates*

Radioactive aspartic acid samples eluted from the chromatograms of the experiments quoted in Figs. 1 and 2 were added to 31 μmoles of non-isotopic aspartic acid. Portions were subjected to total combustion, or decarboxylation with chloramine-T and the transaminase–aniline–citrate system. For details see under Methods. No activity was found in $C_{(2)}+C_{(3)}$.

Substrate: acetate			Substrate: acetaldehyde		
Time of substrate oxidation (min.)	Percentage activity in $C_{(4)}$	Percentage activity in $C_{(1)}$	Time of substrate oxidation (min.)	Percentage activity in $C_{(4)}$	Percentage activity in $C_{(1)}$
10	100	0	20	100	0
30	100	0	30	85	15
60	76	24	60	73	27
120	64	36	120	58	42
150	60	40	150	48	52
180	62	38	180	46	54
210	60	40	—	—	—

Table 7. *Distribution of* ^{14}C *in glutamic acid from* Saccharomyces cerevisiae *after fixation of* $^{14}CO_2$ *with acetaldehyde and acetate as substrates*

Samples of radioactive glutamic acid eluted from the chromatograms of the experiments described in Figs. 1 and 2 were added to 25·5 μmoles of non-isotopic glutamic acid. Portions were subjected to total combustion or decarboxylation with chloramine-T.

Time of substrate oxidation (min.)	Percentage activity in $C_{(1)}$	
	Substrate: acetaldehyde	Substrate: acetate
20	47	—
30	48	83
60	58	66
90	77	70
120	72	80
150	77	—
180	66	94
210	—	83

acids increased after substrate oxidation (Table 5), but this effect was not constant and depends probably on the store of amino groups available for transamination. In controls where only endogenous substrates were oxidized, aspartic and glutamic acid remained constant or slightly diminished.

The specific activities of aspartic and glutamic acid increased continuously throughout the $^{14}CO_2$ fixation, which is due to the progressive labelling of each carbon, as well as the distribution of the isotope in different positions of the molecule. The distribution of ^{14}C in aspartic acid is summarized in Table 6. At first ^{14}C entered only in C-4 but subsequently there was a progressive incorporation of ^{14}C into C-1, which, by the end of the experiment, had nearly half the ^{14}C incorporated. The amino and methylene carbons did not fix the isotope. Aspartic acid labelled during the oxidation of endogenous substrates was also degraded. The distribution of ^{14}C was similar to that shown in Table 6 but the ^{14}C fixed in C-1 never exceeded 30 % of the total.

The distribution of ^{14}C in glutamic acid depends somewhat on the substrate oxidized. With acetaldehyde, only 47–48 % of the ^{14}C incorporated labelled C-1 initially although the proportion increased to 77 % at later stages of incubation. With acetate, more ^{14}C entered C-1, and after 180 min. its activity reached 94 % (Table 7).

Fixation of carbon dioxide by Saccharomyces cerevisiae *with pyruvate as substrate*

Pyruvate metabolism in baker's yeast. Baker's yeast can (1) decarboxylate pyruvate into acetaldehyde, as in glucose fermentation (Meyerhof, 1937), (2) dismute pyruvate to carbon dioxide, acetic and lactic acid (Barron *et al.* 1950), and (3) completely oxidize pyruvate with oxygen (Lieben, 1923) through the citric acid cycle (Barron *et al.* 1950; Wang, Christensen & Cheldelin, 1953). Oxidative decarboxylation to acetate should be the first step of pyruvate oxidation (Barron *et al.* 1950) which is associated with phosphate exchange (Stoppani, 1951) as in the animal enzyme preparations. When baker's yeast metabolizes exogenous pyruvate, the third of these metabolic paths is by far the most important, as shown by the lack of consumption of pyruvate in the absence of oxygen (Barron *et al.* 1950) or in the presence of 2:4- and 2:6-dinitrophenols (Table 8). These substances are strong inhibitors of pyruvate oxidation, but in the same concentrations do not inhibit carboxylase either in the purified state or in the intact yeast cell, as shown by their lack of action on glucose fermentation. Exogenous pyruvate is presumably not accessible to carboxylase and therefore cannot be decarboxylated to acetaldehyde.

Fixation and distribution of $^{14}CO_2$. Baker's yeast incorporates carbon dioxide during pyruvate oxidation (Fig. 7). The amount of ^{14}C fixed is proportional to the oxygen consumption and, during the first 60 min. of incubation, also to the uptake of pyruvate. It is noteworthy that after that time pyruvate apparently disappeared from the yeast suspension, although the amount of oxygen consumed was only 40 % of that required for complete combustion. The close connexion of ^{14}C incorporation and oxygen uptake after incubation for 60 min. shows that in the last period of incubation carbon dioxide fixation was bound more to the oxidation of pyruvate products than to the presence of pyruvate itself. With pyruvate, the rate of fixation is faster than with acetate (or acetaldehyde) as substrate (Fig. 8), which is consistent with the lack of an 'induction period' in pyruvate oxidation.

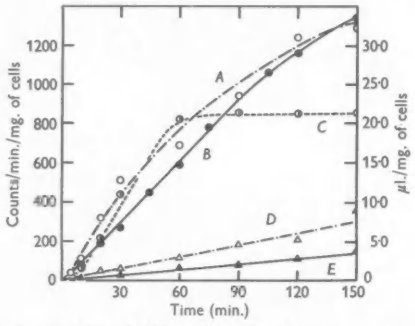

Fig. 7. Fixation of $^{14}CO_2$ and uptake of oxygen and pyruvate by *S. cerevisiae* with pyruvate as substrate. Pyruvic acid 20 mM. Other experimental conditions were as in Fig. 1. *A*, $^{14}CO_2$ fixation with pyruvate and *D*, control; *B*, oxygen uptake with pyruvate and *E*, control; *C*, pyruvate consumption. Pyruvate consumption is expressed in µl. (1 µmole ≡ 22·4 µl.).

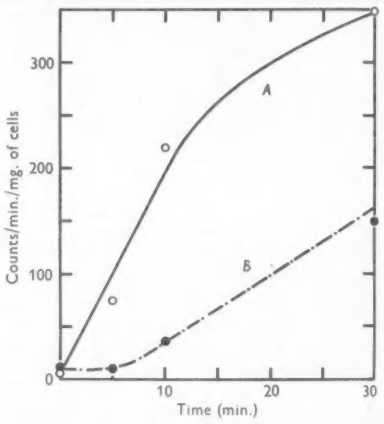

Fig. 8. Comparative rate of $^{14}CO_2$ fixation with pyruvate and acetate as substrates. Yeast: 250 mg.; 5 mM-pyruvic acid (*A*); 5 mM-acetic acid (*B*); pH 3·1; 4·2 µmoles of $NaH^{14}CO_3$ ($3·1 \times 10^6$ counts/min.); 10 ml. final volume. Temp. 30°. Air was in the gas space.

Table 8. *Effect of dinitrophenols on pyruvate oxidation, glucose fermentation and carboxylase activity*

Expt. *A*: 12·1 mg. of yeast; 0·05 M-fumarate; 0·027 M-pyruvate; pH 4·5; incubation for 90 min. Expt. *B*: 3·3 mg. of yeast; 0·05 M-succinate; 0·02 M-pyruvate; pH 4·5; incubation for 90 min. Expt. *C*: 5·5 mg. of yeast; 0·05 M-phosphate; pH 4·5; 0·014 M-glucose; incubation for 60 min. Expt. *D*: 0·3 ml. of carboxylase; 0·05 M-phosphate; 0·03 M-pyruvate; incubation for 10 min. In Expt. *C*, N_2 was in the gas space; air was used in the other experiments. Temp. 30°. The results are expressed in µmoles. 2:4-DNP and 2:6-DNP, 2:4- and 2:6-dinitrophenol.

Expt.		Control	Inhibitor	Inhibition (%)
	Pyruvate oxidation		2:4-DNP (0·1 mM)	
A	CO_2 formation	16·9	0·7	95·8
A	O_2 uptake	14·9	0·9	94·0
A	Pyruvate consumption	16·8	0·1	99·0
	Pyruvate oxidation		2:6-DNP (0·25 mM)	
B	CO_2 formation	14·0	2·2	84·1
B	O_2 uptake	11·2	2·7	76·0
B	Pyruvate consumption	12·9	4·0	69·0
	Glucose fermentation		2:4-DNP (0·1 mM)	
C	CO_2 formation	33·4	31·6	5·4
	Glucose fermentation		2:6-DNP (0·25 mM)	
C	CO_2 formation	33·4	34·7	−3·9
	Carboxylase activity		2:4-DNP (0·1 mM)	
D	CO_2 formation, pH 4·7	94	78	17·0
D	CO_2 formation, pH 6·5	40	40	0·0
	Carboxylase activity		2:6-DNP (0·25 mM)	
D	CO_2 formation, pH 4·8	43	41	4·6

The distribution of $^{14}CO_2$ fixed with pyruvate as substrate is shown in Table 9. No significant amount of ^{14}C was incorporated into the yeast proteins and the distribution in the methanol–water-soluble substances follows, in general, the same pattern as with the C_2 substrates. However, after incubation for 60 min., the dicarboxylic acids (especially malic acid) were far more radioactive than at the same period of the experiments with acetate or acetaldehyde.

The kinetics of the $^{14}CO_2$ fixation with pyruvate (Fig. 9) resemble those with acetate (Fig. 3) with the exception of (*a*) the first period of ^{14}C distribution, which is far shorter with pyruvate (15 min.) and (*b*) the initial positive slope of malic acid. If the absolute distribution of ^{14}C is calculated and plotted against time, it can be shown that, as with acetate, after fixation for 120–180 min. aspartic and glutamic acid are the main reservoirs of the $^{14}CO_2$ incorporated.

^{14}C fixation in aspartic and glutamic acid. During pyruvate oxidation the pools of aspartic and glutamic acid increase; with glutamic acid this occurs at the beginning and with aspartic acid at the end of the experiment. The specific activity of the amino acids increases as well, the variation being particularly marked with glutamic acid (Table 10). ^{14}C first labels the C-4 position of aspartic acid and subsequently C-1 (Table 11), and after incubation for 2 hr. the activity in C-1 approaches 50% of the total fixed. In glutamic acid, ^{14}C labels exclusively C-1 (Table 12), which constitutes a striking difference from the results obtained with acetate or acetaldehyde.

Table 9. *Distribution (%) of $^{14}CO_2$ fixed by* Saccharomyces cerevisiae *with pyruvate as substrate*

Experimental conditions were as in Fig. 7. Total ^{14}C fixed (counts/min./mg. of cells): 641 (incubation for 60 min.) and 1410 (180 min.); ^{14}C fixed in the methanol–water-soluble substances: 652 (60 min.) and 1358 (180 min.).

Substance	^{14}C fixed (% of total in the methanol–water-soluble substances) 60 min.	180 min.
Krebs-cycle intermediates		
Malic acid	18·4	5·1
Citric, aconitic and *iso*citric acid	6·4	3·7
Fumaric acid	2·4	0·5
Succinic acid	2·0	0·5
Amino acids		
Aspartic acid	16·1	48·0
Glutamic acid	44·7	30·3
Alanine	1·0	2·5
Threonine	4·2	1·6
Arginine–ornithine	0·0	1·7
Glycolysis intermediates		
Hexose phosphates	1·4	1·1
Phosphoglyceric acid	0·0	0·3
Phosphopyruvic acid	0·0	2·1
Other substances	3·4	2·6

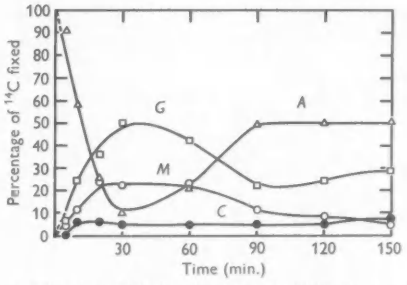

Fig. 9. Kinetics of ^{14}C distribution after $^{14}CO_2$ fixation by *S. cerevisiae* with pyruvate as substrate. Experimental conditions were as in Fig. 7. *A*, Aspartic acid; *G*, glutamic acid; *M*, malic acid; *C*, citric, aconitic and *iso*citric acid.

Table 10. *Concentration and specific activity of free aspartic and glutamic acid in* Saccharomyces cerevisiae *during $^{14}CO_2$ fixation with pyruvate as substrate*

Amounts of amino acids and radioactivity were measured in samples of aspartic and glutamic acid eluted from chromatograms of a fixation experiment similar to that described in Fig. 7, except that the amount of yeast was 500 mg. Amino acids were measured according to Russell (1944).

Time of pyruvate oxidation (min.)	Aspartic acid (μmoles/100 mg. of cells)	Aspartic acid Specific activity (counts/min./μmole)	Glutamic acid (μmoles/100 mg. of cells)	Glutamic acid Specific activity (counts/min./μmole)
2	2·0	425	26·0	7
5	—	—	32·0	11
10	2·0	2700	36·0	20
30	36·0	2360	22·0	162
60	39·0	3220	28·0	373
120	32·0	3100	20·0	778

Table 11. *Distribution of* ^{14}C *in aspartic acid from* Saccharomyces cerevisiae *after fixation of* $^{14}CO_2$ *with pyruvate as substrate*

Radioactive aspartic acid samples eluted from chromatograms of Expts. *A* and *B* were added to 31 μmoles of non-isotopic aspartic acid, and portions were degraded as in Table 6. Conditions of fixation experiments: yeast 490 mg. (Expt. *A*) or 250 mg. (Expt. *B*); pyruvate 10 mM (Expt. *A*) or 5 mM (Expt. *B*). Other details were as in Fig. 1.

Time of pyruvate oxidation (min.)	Percentage activity in $C_{(4)}$		$C_{(1)}$	
	Expt. *A*	Expt. *B*	Expt. *A*	Expt. *B*
5	—	97	—	0
10	91	73	9	27
20	86	—	14	—
30	82	57	18	43
60	69	61	31	39
120	77	56	18	44

Table 12. *Distribution of* ^{14}C *in glutamic acid from* Saccharomyces cerevisiae *after fixation of* $^{14}CO_2$ *with pyruvate as substrate*

Fixation experiment: 490 mg. of yeast and 10 mM-pyruvate. Other experimental details were as in Fig. 1. Radioactive glutamic acid was eluted from the chromatograms, diluted with 25·5 μmoles of non-isotopic glutamic acid and portions were subjected to degradation.

Time of pyruvate oxidation (min.)	Percentage activity in $C_{(1)}$
5	98
10	98
20	86
30	92
120	101

Carbon dioxide fixation by Saccharomyces cerevisiae *with glucose as substrate*

Fixation and distribution of $^{14}CO_2$. In the presence of glucose there is a rapid incorporation of carbon dioxide which greatly exceeds the rate of oxygen uptake. However, this is a transient effect and soon the fixation of ^{14}C slows down to become almost negligible. On the other hand, the velocity of glucose oxidation remains constant throughout the experiment (Fig. 10). The fast initial rate of fixation clearly differentiates the fixation with glucose from the fixation with the other substrates.

Glucose, a non-electrolyte, allowed the effect of the medium pH on the rate of $^{14}CO_2$ fixation to be tested (Table 13). After incubation for 2–5 min. the ^{14}C incorporated at pH 2·8 was less than at pH 7·1, notwithstanding the similar rate of glucose oxidation, but in the course of the incubation the pH of the initially acid suspension increased (up to 6·0) and simultaneously the rate of $^{14}CO_2$ incorporation increased, in such a way that by the end of the experiment the activity fixed by the yeast suspensions was nearly the same. The initial difference may possibly be explained by the lower concentration of HCO_3^- ions at the acid pH.

The distribution of fixed $^{14}CO_2$ is shown in Table 14. It must be noted that the activity incorporated into the cell proteins is significant and that at 5 min. incubation there is strong labelling of malic, fumaric and succinic acid but low activity in aspartic and glutamic acid; this is not observed after short periods of incubation with the other substrates. Nevertheless, if an adequate time of oxidation is allowed, glutamic and aspartic acid regain their place as main reservoirs of ^{14}C and the dicarboxylic acids appear with their usual low percentage of isotope. Other amino acids related to the citric acid-cycle intermediates, such as alanine, glycine, threonine and basic amino acids, fix a small amount of ^{14}C and the activity in the glycolytic

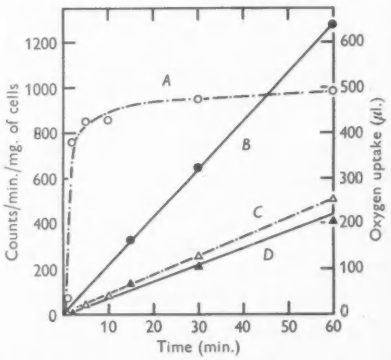

Fig. 10. Fixation of $^{14}CO_2$ and oxygen uptake of *S. cerevisiae* with glucose as substrate. Yeast, 28·5 mg./ml.; 5 mM-glucose and 0·4 mM-phosphate, pH 7·4. Other experimental conditions were as in Fig. 1. *A*, $^{14}CO_2$ fixation with glucose and *C*, control. *B*, Oxygen uptake with glucose and *D*, control.

Table 13. *Effect of initial* pH *on* $^{14}CO_2$ *fixation by* Saccharomyces cerevisiae, *with glucose as substrate*

Yeast, 285 mg.; 0·4 mM-phosphate buffer; 5 mM-glucose. Other experimental conditions were as in Fig. 1 (fixation experiment).

Time of glucose oxidation (min.)	$^{14}CO_2$ fixation (counts/min./mg. of cells)	
	Initial pH 2·8	Initial pH 7·1
2	248	760
5	630	852
30	990	952

Table 14. *Distribution* (%) *of* $^{14}CO_2$ *fixed by* Saccharomyces cerevisiae *with glucose as substrate*

Experimental conditions were as in Fig. 10. Total ^{14}C fixed (counts/min./mg. of cells): 630 (incubation for 5 min.); 832 (10 min.); 994 (30 min.) and 1150 (2 hr.). ^{14}C fixed in the methanol–water-soluble substances: 580 (5 min.) 640 (10 min.) 630 (30 min.) and 850 (2 hr.).

Substance	^{14}C fixed (% of total in methanol–water-soluble substances) 5 min.	10 min.	30 min.	2 hr.
Krebs-cycle intermediates				
Malic acid	31·3	12·5	8·3	3·8
Citric, aconitic and *iso*citric acid	5·7	4·8	2·0	3·5
Fumaric acid	5·0	1·4	1·6	0·5
Succinic acid	2·8	1·4	0·9	0·8
Amino acids				
Aspartic acid	24·5	13·4	38·4	32·7
Glutamic acid	24·4	61·6	43·7	50·8
Alanine	0·7	0·9	1·2	1·5
Glycine	0·0	1·6	0·9	1·2
Threonine	1·4	1·5	0·2	1·1
Arginine–ornithine	0·0	0·0	0·0	0·4
Glycolysis intermediates				
Hexose phosphates	0·0	0·4	0·0	1·1
Phosphoglyceric acid	0·0	0·0	0·0	1·2
Phosphopyruvic acid	0·0	0·5	0·4	0·6
Other substances	4·2	0·0	2·4	0·8

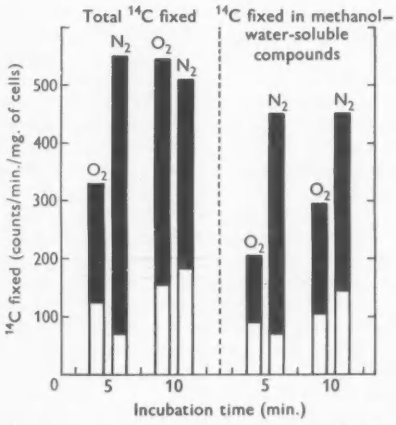

Fig. 11. Aerobic and anaerobic fixation of $^{14}CO_2$ by *S. cerevisiae* with glucose as substrate. Yeast, 22·7 mg./ml.; 5 mM-glucose. Temp. 18°. Other experimental details were as in Fig. 1. Gas exchange with glucose: Q_{O_2}, −16·1; Q_{CO_2}, 15·9 and $Q^{N_2}_{CO_2}$, 19·0; control: Q_{O_2}, −2·6; Q_{CO_2}, 2·6 and $Q^{N_2}_{CO_2}$, 2·3. Shaded areas represent the difference between $^{14}CO_2$ fixed with glucose and the controls.

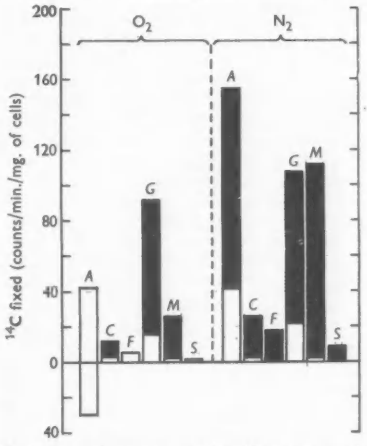

Fig. 12. Action of oxygen on the distribution of $^{14}CO_2$ fixed in methanol–water-soluble substances from *S. cerevisiae* with glucose as substrate. Experimental conditions were as in Fig. 11. Incubation was for 5 min. Shaded areas represent the difference between $^{14}CO_2$ fixed with glucose and the controls (less $^{14}CO_2$ was fixed in aspartic acid when glucose was present). *A*, Aspartic acid; *C*, citric, aconitic and *iso*citric acid; *F*, fumaric acid; *G*, glutamic acid; *M*, malic acid; *S*, succinic acid.

intermediates proves the reversibility of the Embden–Meyerhof sequence of reactions.

The kinetics of $^{14}CO_2$ fixation with glucose are similar to those with pyruvate, as previously shown (Stoppani *et al.* 1957). After 15–30 sec. fixation aspartic acid contains most of the ^{14}C fixed and is the only compound in the distribution plot showing negative slope.

Aerobic and anaerobic fixation of $^{14}CO_2$. Oxygen is not necessary for carbon dioxide fixation in the presence of glucose and, furthermore, after fixation for 5 min. more ^{14}C is incorporated with nitrogen than with oxygen in the gas phase (Fig. 11). The reverse is true in the absence of substrate,

which proves that the anaerobic fixation depends on glycolysis. The distribution of the fixed $^{14}CO_2$ is also affected by the composition of the gas phase. In the first place, oxygen enhances the incorporation of ^{14}C into the cell proteins (Fig. 11). Thus after incubation for 5 min. the percentage of ^{14}C fixed in the cell protein was 40 under oxygen and 21 under nitrogen; after 10 min. the difference was more significant: 51% under oxygen and 6% under nitrogen. Secondly, the distribution of ^{14}C in the methanol–water-soluble substances is also dependent on oxygen, as anaerobically more ^{14}C enters into aspartic, malic, fumaric, succinic and citric acid (Fig. 12).

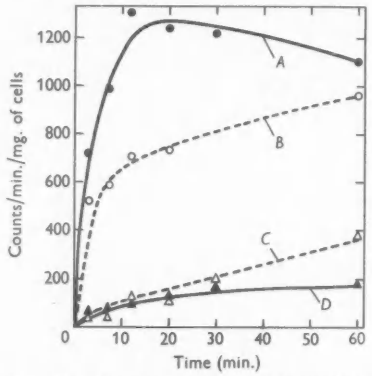

Fig. 13. Action of antimycin A on $^{14}CO_2$ fixation by *S. cerevisiae*. Yeast 12 mg./ml.; 5 mM-glucose; antimycin A, 13 μg./ml.; 4·2 μmoles of $NaH^{14}CO_3$ ($3{\cdot}1 \times 10^6$ counts/min.). Total volume: 2 ml. in the manometric experiment and 6 ml. in the fixation experiment. Temp. 25°. Air was in the gas space. Gas exchange with glucose: Q_{O_2}, −30·7 and Q_{CO_2}, 37·7; with glucose and antimycin A: Q_{O_2}, −1·5 and Q_{CO_2}, 46·2; with antimycin A: Q_{O_2}, −1·5 and Q_{CO_2}, 2.0; control, Q_{O_2} −2·3 and Q_{CO_2}, 3·1. *A*, $^{14}CO_2$ fixation with glucose and antimycin A; *B*, with glucose; *C*, control without and *D*, with antimycin A.

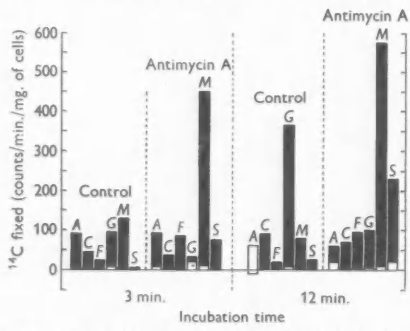

Fig. 14. Action of antimycin A on the distribution of $^{14}CO_2$ fixed by *S. cerevisiae* with glucose as substrate. Details as for Fig. 13 (fixation experiment). The shaded areas represent the difference between the $^{14}CO_2$ fixed with glucose and the controls. *A*, Aspartic acid; *C*, citric, aconitic and *iso*citric acid; *F*, fumaric acid; *G*, glutamic acid; *M*, malic acid; *S*, succinic acid.

Effect of inhibitors. Antimycin A, a selective inhibitor of aerobic cellular oxidation (Ahmad, Schneider & Strong, 1950), brings about changes in $^{14}CO_2$ fixation that resemble those induced by anaerobiosis. With glucose, antimycin A increases the rate of $^{14}CO_2$ fixation, inhibits almost completely the oxygen uptake and promotes evolution of carbon dioxide. On the other hand, in the absence of glucose antimycin A inhibits the $^{14}CO_2$ fixation and oxygen uptake as well (Fig. 13). The distribution of ^{14}C in the methanol–water-soluble substances varies accordingly. With antimycin A there is more labelling of aspartic, malic, fumaric and succinic acid and less of glutamic acid (Fig. 14, 12 min. incubation). Similar changes are observed after incubation for 3 min. except in aspartic acid, the relative labelling of which does not vary.

Two inhibitors of oxidative phosphorylation (2:4-dinitrophenol and sodium azide) and two of glycolysis (fluoride and iodoacetate) have been also tested for their effects on $^{14}CO_2$ fixation. 2:4-Dinitrophenol diminishes the $^{14}CO_2$ incorporation with either glucose or the control but has more effect on the latter (Table 15). As 2:4-dinitrophenol does not interfere with glucose oxidation or fermentation in yeast (Stickland, 1956), it may be assumed that energy-rich phosphate compounds are involved in the fixation reaction. This is confirmed with sodium azide, which strongly inhibits both aerobic and anaerobic fixation of $^{14}CO_2$ (Table 15). The anaerobic inhibition is unexpected, as azide does not inhibit the rate of glucose fermentation and anaerobic phosphorylation by yeast cells (Stickland, 1956) or by yeast extracts (Robertson & Boyer, 1955).

Fixation of ^{14}C *in aspartic and glutamic acid.* The pool and specific activity of glutamic acid varied in the experiments with glucose as with the other substrates. Aspartic acid was not measured. The distribution of ^{14}C in aspartic and glutamic acid is shown in Table 16. ^{14}C is located only in the carboxyl groups of aspartic acid and, at the beginning of the experiment, only in C-4. Subsequently the isotope appears in C-1, which, at the end of the incubation, contained about 40% of the total ^{14}C fixed. In glutamic acid at short time intervals most of the label is located in C-1 but later also in other positions, especially in C-5.

Fixation of ^{14}C *in succinic acid.* Fumaric and succinic acid are labelled during $^{14}CO_2$ fixation. As their isotope distribution is important for the elucidation of the paths of carbon assimilation,

Table 15. *Action of inhibitors on* $^{14}CO_2$ *fixation by* Saccharomyces cerevisiae

Yeast, 14·7 mg.; 0·01 M-glucose; 5·5 μmoles of $NaH^{14}CO_3$ ($1·2 \times 10^6$ counts/min.); 2 ml. of 0·18 M-KH_2PO_4; pH 4·8. Temp. 30°. Corrected fixation: ^{14}C fixed with glucose less control. The percentage inhibition in the experiments with glucose are calculated with the corrected fixation values.

Gas phase	Time of incubation (min.)	Additions	$^{14}CO_2$ fixation (counts/min./mg. of cells) Total	Corrected	Inhibition (%)	CO_2 formation (μl.)
Air	30	Glucose	1830	1010	—	—
Air	30	Glucose + 2:4-dinitrophenol (0·2 mM)	420	360	58·8	—
Air	30	None	820	—	—	—
Air	30	2:4-Dinitrophenol (0·2 mM)	60	—	87·8	—
Air	20	Glucose	2020	1185	—	—
Air	20	Glucose + sodium azide (3·2 mM)	33	29	98·2	—
Air	20	None	835	—	—	—
Air	20	Sodium azide (3·2 mM)	4	—	99·5	—
N_2	20	Glucose	1120	610	—	440
N_2	20	Glucose + sodium azide (3·2 mM)	80	62	89·8	452
N_2	20	None	510	—	—	0
N_2	20	Sodium azide (3·2 mM)	18	—	96·6	30
N_2	30	Glucose	1680	1250	—	290
N_2	30	Glucose + fluoride (5·0 mM)	830	230	81·5	58
N_2	30	Glucose + iodoacetate (0·046 mM)	250	−30	102·2	0
N_2	30	None	430	—	—	—
N_2	30	Fluoride (5·0 mM)	600	—	−39·6	—
N_2	30	Iodoacetate (0·046 mM)	280	—	34·6	—

Table 16. *Distribution of* ^{14}C *in aspartic and glutamic acid from* Saccharamyces cerevisiae *after fixation of* $^{14}CO_2$ *with glucose as substrate*

Samples of radioactive aspartic and glutamic acid eluted from the chromatograms of the experiments summarized in Table 13 (Expt. *A*) or Fig. 10 (fixation Expt. *B*) were added to 31 μmoles of non-isotopic aspartic acid and 25 μmoles of non-isotopic glutamic acid respectively and portions subjected to degradation.

Time of glucose oxidation (min.)	Aspartic acid. Percentage activity in C-4 Expt. *A*	C-4 Expt. *B*	C-1 Expt. *A*	C-1 Expt. *B*	Glutamic acid. Percentage activity in C-1 Expt. *A*	C-1 Expt. *B*	C-5 Expt. *B*
2	94	91	6	9	89	—	—
5	72	77	28	23	81	79	21
10	71	56	29	44	77	78	22
20	79	—	21	—	70	—	—
30	66	60	34	40	71	63	25
60	69	—	31	—	67	59	27
120	61	—	39	—	61	—	—

Table 17. *Distribution of* ^{14}C *in succinic acid formed by* Saccharomyces cerevisiae *in the presence of glucose and antimycin* A

Samples of succinic acid eluted from the chromatograms of the fixation experiment quoted in Fig. 13 were added to 23·3 mg. of non-isotopic succinic acid and portions subjected to combustion or decarboxylation. Specific activities are in counts/min./mg. of $BaCO_3$ and the probable error of the mean is quoted.

Time of glucose oxidation (min.)	Specific activity of succinic acid carbon atoms $^{14}CO_2H$	$^{14}CH_2$	Percentage activity in CO_2H
7	3·5 ± 0·2	−0·1 ± 0·3	103 ± 4·6
12	7·2 ± 0·4	−0·1 ± 0·4	101 ± 4·2
20	6·8 ± 0·5	−0·6 ± 0·6	109 ± 6·2

samples of radioactive succinic acid from the experiment of $^{14}CO_2$ fixation with glucose and antimycin A were degraded. Table 17 shows that only the carboxyl carbons were radioactive.

Fixation of carbon dioxide by Saccharomyces carlsbergensis

The relative deficiency of oxidizing mechanisms does not prevent the assimilation of carbon dioxide by *S. carlsbergensis* (Table 18). Glucose and lack of oxygen stimulate the isotope incorporation, the distribution of which (Table 19) shows the same pattern as that with baker's yeast under anaerobic conditions, namely, a comparatively strong labelling of malic, fumaric and succinic acid and low activity in glutamic acid.

Table 18. *Carbon dioxide fixation by* Saccharomyces carlsbergensis

Yeast, 7·5 mg.; 5 mM-glucose; 1·6 μmoles of $NaH^{14}CO_3$ ($1·2 \times 10^6$ counts/min.). Final volume, 2 ml. Incubation was at 20° for 20 min.

Gas phase	Additions	$^{14}CO_2$ fixation (counts/min./mg. of cells)	CO_2 formation (μl.)	Oxygen uptake (μl.)
N_2	None	950	40	—
N_2	Glucose	2000	160	—
Air	None	350	60	28
Air	Glucose	1600	220	30

Table 19. *Distribution of* $^{14}CO_2$ *fixed by* Saccharomyces carlsbergensis *with glucose as substrate*

Experimental conditions were as in Table 18. ^{14}C fixed in the methanol–water-soluble substances (counts/min./mg. of cells): 1600 (with glucose) and 300 (control). Corrected distribution (%) of ^{14}C: total ^{14}C fixed with glucose less the respective control value, %.

Substance	Total ^{14}C fixed (counts/min./mg. of cells)		Percentage distribution of ^{14}C with glucose	
	With glucose	Control	Found	Corrected
Krebs-cycle intermediates				
Malic acid	510	105	32·0	31·2
Citric, aconitic and *iso*citric acid	48	5	3·0	3·3
Fumaric acid	96	27	6·0	5·3
Succinic acid	152	63	9·5	6·8
Amino acids				
Aspartic acid	480	66	30·0	32·0
Glutamic acid	107	21	6·7	6·6
Alanine	91	9	5·7	6·3
Threonine	40	0	2·5	3·1
Glycolysis intermediates				
Phosphopyruvic acid	26	0	1·6	2·0
Other substances	48	4	3·0	3·4

DISCUSSION

Of the primary reactions for the incorporation of carbon dioxide into cell metabolism (Utter & Wood, 1951) only the formation of dicarboxylic acids by β-carboxylation provides a satisfactory explanation for carbon dioxide assimilation by yeasts. The synthesis of dicarboxylic acids can be performed by (1) reductive carboxylation of pyruvate, which yields malate (Ochoa, Mehler & Kornberg, 1948), (2) carboxylation of phosphoenolpyruvate, which yields oxaloacetate (Utter & Kurahashi, 1954; Bandurski & Lipmann, 1956), and (3) carboxylation of propionate, which yields succinate (Lardy & Adler, 1956). In yeasts, only the second mechanism seems to be valid, even if oxaloacetic acid, on account of instability, does not appear on the chromatograms. In fact, oxaloacetic acid being the primary fixation compound, the curves of ^{14}C distribution in its immediate derivatives, aspartic, malic and citric acid, will at first show a positive slope following the 'mammillary' model of the kinetics of isotope distribution (cf. Whitehouse & Putman, 1953). However, if one of the secondary reactions (e.g. the transamination of oxaloacetic acid) were much faster than the other two, the maximum of the respective curves may be reached before the isotope distribution could be measured and then the curve of aspartic acid would appear as having a negative slope. On the other hand, if carbon dioxide is incorporated through malic or succinic acid the isotope fixed should follow, totally or in part, the sequence succinic, fumaric, malic, oxaloacetic, aspartic (or citric), that is, a 'catenary' model of isotope distribution (cf. Whitehouse & Putman, 1953) and, accordingly, the curve of succinic (or malic) acid would be the first to have a negative slope. In general the experimental data fulfil the requirements for carbon dioxide fixation through oxaloacetic acid, but with acetate or acetaldehyde the curve of malic acid shows a small negative slope. This, however, if significant does not oppose the role of oxaloacetic acid as the first product of carbon dioxide fixation and can be easily explained by the delay in recording ^{14}C fixation and distribution in the experiments with C_2 substrates. The 'induction' period of oxidation

prevents the measurement of ^{14}C distribution at a time interval short enough to permit the plotting of the malic acid curve before it reaches its maximum.

All the experimental evidence supports phosphopyruvic acid as the primary carbon dioxide acceptor. Phosphopyruvic acid is formed in anaerobic glycolysis (Meyerhof, 1937), a process inhibited by iodoacetate and fluoride; by oxidation of Krebs-cycle intermediates (Kalckar, 1939; Leloir & Muñoz, 1944) and by direct phosphorylation of pyruvic acid (Lardy & Ziegler, 1945). An effective carbon dioxide fixation takes place under experimental conditions involving any of the above-mentioned reaction mechanisms. Thus (1) with glucose as substrate the fastest fixation occurs under anaerobic conditions and is inhibited by iodoacetate and fluoride; (2) during acetate or acetaldehyde oxidation the amount of ^{14}C incorporated is proportional to the amount of substrate metabolized, in other words to the extent of the Krebs-cycle operation, and (3) phosphoenolpyruvic acid can be carboxylated by cell-free extracts of baker's yeast, which also contain pyruvic phosphokinase (observations with J. Cannata, unpublished work). This explains why, after a short incubation, the ^{14}C incorporation and oxygen consumption are faster with pyruvate than with acetate, as the first forms directly phosphopyruvate which immediately promotes carbon dioxide fixation and consequently, the synthesis of the oxaloacetate required to set in operation the cycle. With acetate, except for other less efficient mechanisms such as endogenous-substrate oxidation, phosphopyruvate and oxaloacetate must be formed through the Krebs cycle, that is, the same process they are supposed to start. The carboxylation of pyruvate is also consistent with the distribution of ^{14}C in succinic acid formed by living yeast cells from [2-^{14}C]pyruvate (Stoppani, de Favelukes & Conches, 1958).

The location of ^{14}C in aspartic acid fits in with the proposed C_3+C_1 condensation reaction. Thus after short time fixation all the isotope concentrates in C-4 which reflects the isotope concentration of the β-carboxyl group of oxalacetate. Later, also C-1 of aspartic acid incorporates ^{14}C and this is explained by the partial equilibration of oxaloacetic acid with malic acid, and through the latter with the symmetrically labelled fumaric acid. This interpretation is supported by the distribution of ^{14}C in the succinic acid isolated from the experiments of $^{14}CO_2$ fixation in the presence of glucose and antimycin A. Further, it is still possible that aspartic acid equilibrates directly with fumaric acid, as aspartase has been reported in yeast (Haen & Leopold, 1937). The direct relationship of aspartic acid with the first product of $^{14}CO_2$ fixation, its double labelling and the increase of its pool after sufficient substrate oxidation has occurred account for the large amount of ^{14}C stored in aspartic acid and, consequently, for the peculiar pattern of its curve in the distribution plots.

With pyruvate as substrate, the incorporation of ^{14}C in glutamic acid confirms the fixation path predicted, as the label is located in C-1. This is consistent with the distribution of ^{14}C in aspartic acid (and therefore in oxaloacetate), the synthesis of α-oxoglutarate through the reactions of the Krebs cycle and the asymmetrical behaviour of the citric acid molecule (cf. Krebs, 1954). The concentration of ^{14}C in C-1 agrees also with the distribution of ^{14}C found by Wang *et al.* (1953) in glutamic acid formed by *S. cerevisiae* from [2-^{14}C]pyruvate. With glucose as substrate, only in the initial stages of the fixation does ^{14}C concentrate in C-1 of glutamic acid, and later a significant proportion appears in C-5. Also, with acetaldehyde and acetate, a relatively low labelling of C-1 is found, but in this case the abnormal distribution is already evident at the beginning of the experiment, especially with acetaldehyde. Nevertheless, even with these substrates, after prolonged incubation most of the ^{14}C fixed in glutamic acid still appears in C-1, which proves that the reactions leading the isotope to other carbons are of secondary importance. The labelling of C-5 of glutamic acid suggests previous formation of carboxyl-labelled acetate and resembles the synthesis of glutamic acid obtained with some preparations of rat liver (Plaut & Lardy, 1951), hen oviduct (Hendler & Anfinsen, 1954) or *Clostridium kluyveri* (Tomlinson, 1954). Whether similar reactions take place in baker's yeast cannot be ascertained with the data available. Further studies (now in progress) of ^{14}C distribution in the amino acids formed by *S. cerevisiae* from [1-^{14}C]- and [2-^{14}C]-acetate may elucidate the point.

The Krebs-cycle model is the mechanism that better explains the distribution of the $^{14}CO_2$ fixed during substrate oxidation by *S. cerevisiae*, as well as the distribution of ^{14}C in succinic acid formed by this organism from [1-^{14}C]-, [2-^{14}C]-acetate and [2-^{14}C]pyruvate (Stoppani *et al.* 1958). As the nature of the main path of substrate oxidation in baker's yeast has been the subject of a long debate, before closing its discussion it seems worth while to consider other metabolic routes, the possibility of whose existence is indicated by recent studies. Thus the glyoxylate cycle (Kornberg & Krebs, 1957; Kornberg & Madsen, 1957), or the direct oxidation of acetate through glyoxylate, is consistent with the presence of *iso*citratase in baker's yeast (Olson, 1954) and the trapping experiments of Bolcato, de Benard & Leggiero (1957) respectively. However, none of these

mechanisms can account for (1) the strong labelling of glutamic acid after the fixation of $^{14}CO_2$ (Davis *et al.* 1956, and the present study) or the oxidation of [^{14}C]acetate (Labbe, Thomas, Cheldelin, Christensen & Wang, 1952 and our own unpublished observations); (2) the insignificant appearance of glyoxylic acid in the chromatograms of yeast extracts after fixation of $^{14}CO_2$ or the oxidation of [^{14}C]-acetate (unpublished); and (3) the lack of isotope incorporation into glyoxylate by yeast preparations oxidizing [^{14}C]acetate (Krebs *et al.* 1952). This does not rule out glyoxylic acid as an intermediate in yeast metabolism since this compound may well intervene in the synthesis of glycine. In support of this possibility it must be recalled that the glyoxylic acid formed from *iso*citric acid labelled after $^{14}CO_2$ fixation is radioactive and that glycine appears consistently among the amino acids which incorporate ^{14}C.

To appraise the role of carbon dioxide fixation in yeasts it must be considered in connexion with the metabolism of glucose, i.e. their main source of carbon and energy. Glucose is degraded through the Embden–Meyerhof path (Eaton & Klein, 1957) to pyruvate and this is then decarboxylated and oxidized to acetyl-coenzyme A which, in the presence of oxaloacetate, yields citrate. Under aerobic conditions, citrate can be completely oxidized in order to liberate energy, but if nitrogen compounds are available, citrate as well as oxaloacetate is assimilated and provides the carbon skeletons for many amino acids. Both the oxidation and assimilation processes are dependent on carbon dioxide fixation because this reaction is the main supply of the oxaloacetate required to initiate and maintain the Krebs cycle, to replace the carbon drained into the amino acid pools and to replace the oxaloacetate destroyed by a carboxylase present in baker's yeast (observations with J. Cannata, unpublished work) which resembles the oxaloacetate carboxylase from *Micrococcus lysodeikticus* (Krampitz & Werkman, 1941). The relationship between assimilation processes and carbon dioxide fixation is further supported by their similar inhibition with azide and 2:4-dinitrophenol, two selective poisons of carbon assimilation (Clifton, 1946).

Under anaerobic conditions (this case includes *S. carlsbergensis*), the degradation of glucose to pyruvate also follows the Embden–Meyerhof path, but the rate of the reactions depends on the concentration of organic hydrogen acceptors and, among these, the fumarate–succinate system is one of the more powerful oxidants. The efficiency of that system is enhanced by the continuous excretion of succinic acid into the medium (Conway & Brady, 1950) which keeps its intracellular concentration at a low level. In the presence of nitrogen compounds, assimilation reactions are also possible and the carbon of citrate and oxaloacetate is turned into amino acids, consequently diminishing the synthesis of succinic acid (Kleinzeller, 1941). Again, carbon dioxide fixation is essential for oxidation and assimilation reactions as, through furnishing oxaloacetate, it provides C_4 hydrogen acceptors and carbon skeletons for amino acids.

SUMMARY

1. The mechanism of carbon dioxide fixation by the yeasts *Saccharomyces cerevisiae* (baker's yeast) and *Saccharomyces carlsbergensis* has been studied.

2. Baker's yeast assimilates carbon dioxide during the oxidation of acetate, acetaldehyde, pyruvate and glucose, and also during glucose fermentation, but the oxidation of endogenous substrates is far less effective.

3. After incorporation of radioactive carbon dioxide, ^{14}C labels the intermediates of the Krebs cycle, a number of amino acids and compounds related to glycolysis. Aspartic, glutamic, malic and the tricarboxylic acids are the main reservoirs of the ^{14}C fixed. The distribution patterns vary somewhat according to the substrate oxidized.

4. Wide variations in pH of the medium do not significantly affect the fixation and distribution of radioactive carbon dioxide.

5. Oxaloacetate is believed to be the primary product of fixation and its formation to take place by carboxylation of phosphoenolpyruvate.

6. The patterns of ^{14}C distribution and the location of ^{14}C in aspartic, glutamic and succinic acid are, as a rule, consistent with the Krebs-cycle model.

7. In the presence of glucose, carbon dioxide fixation is inhibited by 2:4-dinitrophenol, sodium azide, iodoacetate and fluoride, and increased by antimycin A. The fixation dependent on endogenous substrate oxidation is inhibited by 2:4-dinitrophenol, sodium azide, iodoacetate and antimycin A, and increased by fluoride.

8. With glucose as substrate, aerobic and anaerobic patterns of fixation differ. In the absence of oxygen (or inhibition of oxidizing mechanisms with antimycin A) more ^{14}C enters into malic, fumaric, succinic and aspartic acid. In *S. carlsbergensis*, the distribution of ^{14}C belongs to the anaerobic type.

9. Carbon dioxide fixation is an essential reaction for yeast metabolism as it supplies carbon for biosynthetic processes and promotes aerobic and anaerobic oxidations.

10. Baker's yeast consumes exogenous pyruvate mainly by oxidation.

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The Production of Secondary Potassium Depletion, Sodium Retention, Nephrocalcinosis and Hypercalcaemia by Magnesium Deficiency

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Studies of dietary magnesium depletion in the rat have been made by Kruse, Orent & McCollum (1932), Watchorn & McCance (1937) and Tufts & Greenberg (1938). These workers reported an early stage of vasodilatation and hyperexcitability followed by a chronic stage of cachexia. Little or no change was found in the magnesium content of the tissues other than bone, teeth and serum (Watchorn & McCance, 1937), although Tufts & Greenberg (1938) reported minor changes in muscle and brain.

The failure of magnesium-deficient diets to produce changes of magnesium content in the soft tissues contrasts with the marked decreases in muscle potassium produced by potassium deficiency (Heppel, 1939). For this reason it was considered that a fuller investigation of magnesium deficiency was necessary. This has been undertaken by using specific flame-spectrophotometric techniques.

EXPERIMENTAL

Thirty white female rats were placed in two equal groups. The control group (mean initial wt. 101 g.) and the experimental group (mean initial wt. 99 g.) were fed on diets identical in respects other than magnesium content. Rats from each group were killed at intervals by exsanguination under ether anaesthesia.

Ten rats from the control group were killed, at 11, 21, 28, 35, 42, 49, 54, 63 and 70 (two rats) days. None of the control group died. Eight rats from the experimental group were killed at 11, 21, 28, 35, 42, 49, 54 and 62 days. The remaining seven rats of this group died (see Results).

Diet. The diets were made by mixing acid-washed casein (200 g.), arachis oil (80 g.), cod-liver oil (20 g. to which had been added 50 mg. of vitamin E), cane sugar (660 g.) and either salt-mixture C (40 g., control group) or salt-mixture D (36·7 g., experimental group).

Salt-mixture C consisted of NaCl (33 g.), $CaCO_3$ (188 g.), KH_2PO_4 (170 g.), potassium citrate (61 g.), ferric citrate (7·5 g.), 'trace mixture' (1 g.) and $MgCl_2,6H_2O$ (41 g.). In salt mixture D the $MgCl_2$ was omitted.

The 'trace mixture' consisted of KI (13 g.), NaF (10 g.), $MnCl_2,4H_2O$ (2 g.) and Cu_2Cl_2 (0·5 g.).

A stock vitamin solution was prepared and stored at −20°; 250 ml. contained vitamin B_1 (0·03 g.), vitamin B_6 (0·03 g.), biotin (0·006 g.), pantothenol (0·3 g.), *p*-aminobenzoic acid (1·5 g.), inositol (1·5 g.), nicotinic acid (sodium salt; 1·5 g.), choline chloride (4·5 g.), folic acid (0·015 g.), riboflavin (0·12 g.), vitamin B_{12} (0·0003 g.) and vitamin K analogue (Synkavit, Roche Products Ltd., Welwyn Garden City, Herts.; 0·0006 g.).

This solution (16 ml.), made to 2 l. with water, provided sufficient drinking water and water-soluble vitamins for 30 rats for 3 days.

The diets were given *ad lib.* The electrolyte content of the diets (m-equiv./kg.) as found by analysis was: Na, 46; K, 141; Ca, 300; Mg, 36 (control group) and 1·0 (deficient group).

Flame-photometric procedure. Na, K, Ca and Mg were determined in solutions of tissue and plasma ash by flame spectrophotometry at wavelengths of 589, 404, 423 and 285 mμ respectively. Chloride was determined in dry muscle powder and in plasma by measuring the excess of silver flame photometrically at 328 mμ after precipitation of silver chloride (Menis, House & Rains, 1957). Appropriate measures previously described were taken to eliminate interferences (MacIntyre, 1957). The instrument was modified as follows: the earlier burner was replaced by an integral atomizer-burner of the type used in the Beckman flame spectrophotometer (Beckman Instruments Inc., South Pasadena, California, U.S.A.) and described by Burriel-Martí & Ramírez-Muñoz (1957). The range of the instrument was extended to the ultraviolet by an Uvispek monochromator (Hilger and Watts Ltd., London N.W. 1) and quartz-window photomultiplier (type 6256B, E.M.I. Electronics Ltd., Hayes, Middlesex). An oxyhydrogen flame was used except for magnesium. With the authors' instrument satisfactory estimation of magnesium was possible only with oxyacetylene. Under these conditions the interferences were small. With a solution containing 0·5 m-equiv. of Mg/l. and 35 m-equiv. of Na/l. the proportions of the total emission at 285 mμ due to Mg, Na and the flame were 56, 2 and 42% respectively. Phosphate did not interfere.

Sampling techniques

Muscle. Samples were obtained from the thighs, and dried to constant weight at 105° and then ground in a mortar with several changes of a mixture of equal volumes of ethyl ether and light petroleum (b.p. 40–60°). Obvious tendon was removed. The powder was replaced in the oven at 105° for 12 hr. and stored in a desiccator. Duplicate samples (20 mg.) of the dry fat-free muscle powder were weighed into crucibles and ashed at 400°. The ash was dissolved in N-HCl (4 ml./20 mg. of muscle powder) for flame photometry.

Duplicate samples (15 mg.) of the muscle powder were weighed into accurately graduated conical centrifuge tubes for chloride analysis. A volume (1 ml.) of 8N-HNO_3 solution containing 2 m-equiv. of Ag/l. was added and the

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tubes were heated in a boiling-water bath for 30 min. After cooling, the volumes were made to 4 ml. and the tubes centrifuged. Silver was then determined in the clear supernatant and the chloride content of the original sample calculated.

Collagen was determined in duplicate samples (10 mg.) by the method of Neuman & Logan (1950) but the preliminary extraction with urea solution was omitted.

A further group of 12 female rats (approx. wt. 100 g.) on stock diet were killed by exsanguination (six rats) or by a blow on the head and samples of muscle from the thigh and back obtained for analysis. The carcasses were allowed to remain at room temperature for 18 hr., when second samples of muscle were obtained from each rat. Collagen was not estimated in this group.

Plasma. Heparin was used as anticoagulant. Plasma was separated immediately: 0·02 g. was taken for chloride analysis by the method described for muscle, 0·5–2·0 g. was dried and ashed in platinum crucibles and the ash was taken up in N-HCl (4 ml./g. of plasma) for flame photometry.

Bone. A complete femur was analysed. It was ground, defatted as for muscle and the total dry fat-free weight recorded before ashing with a mixture of equal volumes of HNO_3 and $HClO_4$. After evaporation to dryness the ash was dissolved in N-HCl (100 ml./g. dry fat-free wt.) and analysed for Ca and Mg.

Brain, liver and kidney. These were analysed for Ca, Mg and K by the method used for muscle, after ashing as for bone, except that fat was not extracted from brain.

Statistical analysis. The results were analysed as described by Fisher (1950).

RESULTS

During the first 10 days of the experiment both the control and deficient group failed to grow. Subsequently the controls grew rapidly while the deficient group grew very little and finally lost weight (Fig. 1). After about 12 days the deficient rats developed vasodilatation and oedema of the nose, ears and paws. This lasted for 7–10 days.

Seven of the deficient rats died after 39, 42, 43, 54, 62 (two) and 64 days. In five, samples of bone and muscle were analysed, since they were obtained within a few hours of death. The carcasses of the rats dying at 43 and 54 days were not analysed.

Table 1 shows that the Mg and Ca content of muscle remains constant for 18 hr. after death. Differences between the electrolyte concentrations in Tables 1 (*a*) and 2 (*c*) may be attributable to differences in age of animal (Holliday, Segar, Lukenbill, Valencia & Durell, 1957) and collagen content of sample.

Ion changes in different tissues

Muscle. There was a progressive fall in Mg content of muscle in the deficient group with a highly significant correlation ($r = 0{\cdot}72$, $P < 0{\cdot}01$) between muscle Mg and time on deficient diet. The data were expressed by the equation: muscle Mg (m-equiv./kg. of dry fat-free solids) = 102·6 − 0·353 (days on deficient diet). After 64 days

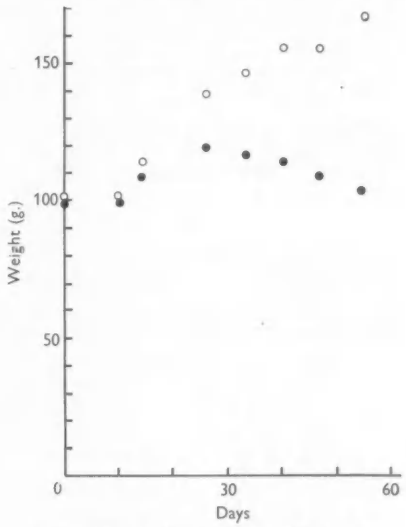

Fig. 1. Growth of control and deficient rats. ●, Deficient; ○, control.

Table 1. *Post-mortem changes in muscle-electrolyte content*

Samples of muscle from thigh and back were obtained from 12 rats, which were then killed. The carcasses were allowed to remain at room temperature for 18 hr., when further samples were obtained from the opposite thigh and opposite side of the back. The figures are m-equiv./kg. of dry fat-free solids. Changes in Ca and Mg are not significant.

	Pre-mortem concn. (mean ± S.E.) (*a*)	Post-mortem concn. (mean ± S.E.) (*b*)	Mean difference ± S.E.M. difference* (*b* − *a*)	*P* (*t* test)
Na	106·1 ± 2·8	128·6 ± 4·6	22·5 ± 5·3	<0·005
K	500·5 ± 6·0	462·6 ± 3·5	−37·9 ± 5·3	<0·001
Ca	10·35 ± 0·19	10·56 ± 0·40	0·21 ± 0·33	>0·5
Mg	103·5 ± 1·3	100·8 ± 2·7	−2·7 ± 1·4	>0·05
Cl	72·5 ± 1·5	122·3 ± 2·7	49·8 ± 3·1	<0·001

* The S.E.M. difference was calculated from the difference in each animal.

the Mg had fallen to 78% of the initial value (Fig. 2).

An unexpected and striking finding was a decline in muscle K (Fig. 3). A highly significant correlation between muscle Mg and K was found ($r=0.93$, $P<0.01$), those animals which died during the experiment being excluded from this calculation. Results showed a fall in muscle K to 80% of the initial value after 64 days and were described by the equation: muscle K (m-equiv./kg. of dry fat-free solids) = 73 + 3·55 (muscle Mg). After correction for post-mortem changes (Table 1)

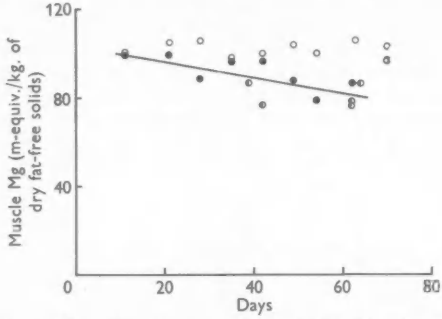

Fig. 2. Magnesium content of muscle. The line is the regression of Mg on time (see text). ●, Deficient rats; ◐, deficient animals which died; ○, control rats.

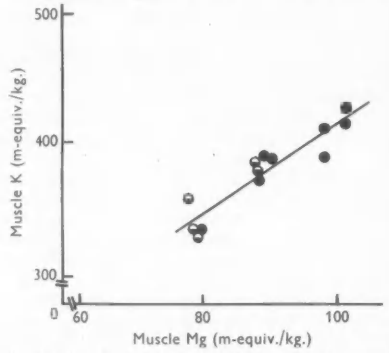

Fig. 3. Relationship of muscle Mg and K. The muscle K contents of the animals which died were corrected for post-mortem fall (Table 1) and are plotted on the graph. ●, Deficient rats; ◒, deficient animals which died.

Table 2. *Electrolyte changes in muscle*

Electrolytes are expressed as m-equiv./kg. of dry fat-free solids. Intracellular Na (Na_i) was calculated as total muscle $Na - \left\{\frac{Na/kg.\ of\ plasma}{Cl/kg.\ of\ plasma} \times 0.94 \times Cl/kg.\ of\ muscle\ dry\ fat\text{-}free\ solids\right\}$*. Samples taken from those animals which died are included only for Ca and Mg. For Mg and K in the deficient group the standard errors were calculated by analysis of variance, taking into account the regressions shown in Figs. 2 and 3 respectively.

	(*c*) Control		(*d*) Deficient			
	Days on diet. Mean ± S.E. (no.) range	Mean (± S.E.M.)	Days on diet. Mean ± S.E. (no.) range	Mean (± S.E.M.)	Mean diff. (± S.E.M. diff.)	*P* (*t* test)
Na	44 ± 6 (10) 11–70	91·7 ± 2·9	38 ± 6 (8) 11–62	142·8 ± 7·8	51·1 ± 8·3	<0·001
K	44 ± 6 (10) 11–70	444·3 ± 8·8	38 ± 6 (8) 11–62	396·1 ± 3·7	−48·2 ± 9·6	<0·001
Ca	44 ± 6 (10) 11–70	14·1 ± 2·6	44 ± 5 (13) 11–64	18·7 ± 2·5	4·6 ± 3·6	>0·05
Mg	44 ± 6 (10) 11–70	100·9 ± 1·0	44 ± 5 (13) 11–64	87·1 ± 1·6	−13·8 ± 1·9	<0·001
Cl	44 ± 6 (10) 11–70	59·5 ± 3·6	38 ± 6 (8) 11–62	94·1 ± 3·2	34·6 ± 4·8	<0·001
Na_i	48 ± 6 (9) 21–70	12·7 ± 2·4	42 ± 6 (7) 21–62	11·1 ± 4·9	−1·6 ± 5·5	>0·5
Collagen (% dry fat-free solids)	44 ± 6 (10) 11–70	3·7 ± 0·2	45 ± 5 (13) 11–64	5·7 ± 0·4	2·0 ± 0·4	<0·001

* The factor 0·94 corrects for the difference between plasma water and interstitial-fluid concentrations of Na and Cl due to the Donnan effect. For other assumptions see text.

the results from the animals which died fit the equation (Fig. 3). No such correlation existed in the control group.

The mean electrolyte changes are summarized in Table 2. It will be seen that in the deficient group there were large increases of total Na and Cl, of 51·1 and 34·6 m-equiv./kg. respectively. If the assumption is made that chloride is entirely extracellular (Manery, 1954) the proportion of the total Na which is intracellular can be calculated from a knowledge of the plasma concentration. No difference was detected in 'intracellular' sodium when calculated by this method. This indicates that the extra sodium in the magnesium-deficient animals was entirely extracellular or was accompanied intracellularly by chloride. In neither case could Na have replaced the K lost.

Table 3. *Electrolyte changes in the viscera*

Each value is the mean (no.) ± S.E.M. expressed as m-equiv./kg. of dry fat-free solids except for brain, where the figures are expressed as m-equiv./kg. of dry solids. The mean time on the deficient diet was 38 days. The increase in Ca in the kidneys of the deficient rats is highly significant ($P<0·01$, t test). The other differences are not significant.

	Kidney		
	Control (*a*)	Deficient (*b*)	Mean diff. (± S.E.M. diff.) (*b* − *a*)
Mg	82·7 (10) ± 1·2	78·8 (8) ± 2·1	−3·9 ± 2·3
K	303·0 (10) ± 3·4	309·3 (8) ± 8·1	6·3 ± 8·7
Ca	12·4 (10) ± 0·5	52·5 (8) ± 13·4	40·1 ± 13·4
	Liver		
	Control (*c*)	Deficient (*d*)	Mean diff. (± S.E.M. diff.) (*d* − *c*)
Mg	81·7 (10) ± 2·5	80·9 (8) ± 1·6	−0·8 ± 3·0
K	352·5 (10) ± 3·0	362·3 (8) ± 5·7	9·8 ± 6·4
Ca	5·2 (9) ± 0·2	5·0 (8) ± 0·2	−0·2 ± 0·3
	Brain		
	Control (*e*)	Deficient (*f*)	Mean diff. (± S.E.M. diff.) (*f* − *e*)
Mg	75·4 (8) ± 1·3	72·4 (7) ± 1·5	−3·0 ± 2·0
K	460·5 (8) ± 4·5	467·3 (7) ± 6·6	6·8 ± 8·1
Ca	19·1 (8) ± 3·1	17·9 (7) ± 1·8	−1·2 ± 3·6

Table 4. *Electrolyte changes in plasma*

Figures are m-equiv./kg. of plasma. The mean time on the deficient diet was 42 days, except for those animals whose plasma was analysed for K, in which it was 32 days.

	Control (*c*) Mean ± S.E. (no.) range	Deficient (*d*) Mean ± S.E. (no.) range	Mean diff. (± S.E.M. diff.) (*d* − *c*)	*P* (*t* test)
Na	140·4 ± 1·5 (9) 135–147	150·4 ± 2·7 (7) 137–157	10·0 ± 3·1	<0·01
K	5·5 ± 1·0 (4) 3·9–8·4	5·2 ± 0·8 (4) 3·5–6·7	−0·3 ± 1·3	>0·5
Ca	5·3 ± 0·1 (9) 4·9–6·0	6·4 ± 0·3 (7) 3·9–8·0	1·1 ± 0·3	<0·01
Mg	1·74 ± 0·09 (9) 1·38–2·16	0·79 ± 0·10 (7) 0·41–1·07	−0·95 ± 0·13	<0·001
Cl	99·2 ± 1·7 (9) 93–107	99·9 ± 3·0 (7) 90–115	0·7 ± 3·3	>0·5

The slight increase in collagen content in the deficient group was too slight to affect interpretation of the other changes.

Kidney, liver and brain. Concentration of Mg did not differ significantly in the two groups in these tissues. Calcium was markedly increased in the kidneys of the deficient animals but normal in the liver and brain (Table 3 and Fig. 5).

Plasma. The deficient group showed reduction in Mg, a slight increase in Na and a marked increase in Ca (Table 4). Fewer analyses were made of K content but no consistent change was seen. Fig. 4 shows that in the deficient group the Mg concentration fell to about 0·4 m-equiv./kg. within 3 weeks and thereafter increased slightly. In Fig. 5 plasma Ca is plotted against renal Ca. Nephrocalcinosis occurred only in the presence of hypercalcaemia.

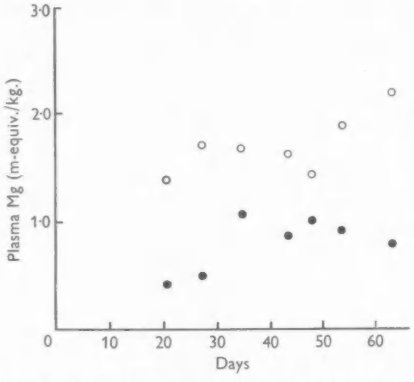

Fig. 4. Plasma Mg concentration. ●, Deficient rats; ○, control rats.

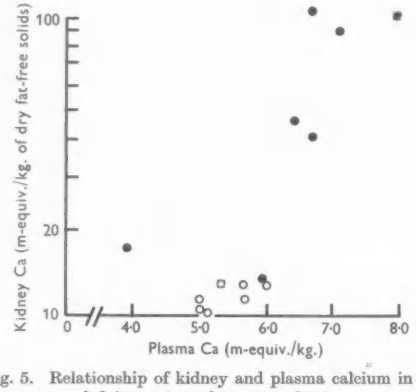

Fig. 5. Relationship of kidney and plasma calcium in ●, deficient rats and ○, control rats.

Bone. Within 3 weeks there was a decline in Mg concentration to about half that of the control group (Fig. 6). Subsequently the concentration declined less rapidly. Fig. 7 shows that during this latter phase the total Mg in the femurs of deficient animals altered very little. The dry fat-free weights of femurs from control and deficient animals were 236 and 234 mg. respectively at 21 days, and 340 and 280 mg. respectively at 64 days.

DISCUSSION

Kruse *et al.* (1932) were the first to describe the characteristic signs of magnesium deficiency in rats. These consist of peripheral vasodilatation and

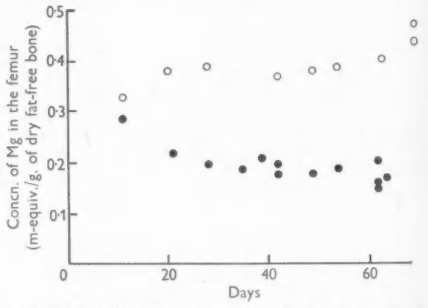

Fig. 6. Magnesium concentration of the femur in ●, deficient rats and ○, control rats.

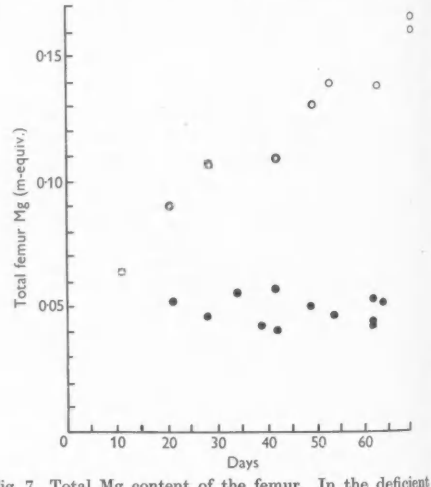

Fig. 7. Total Mg content of the femur. In the deficient animal killed after 11 days total Mg could not be calculated as a portion of the femur was lost. ●, Deficient rats; ○, control rats.

oedema occurring after some days on a deficient diet. They used rats weighing about 50 g. and observed hyperexcitability and convulsions after about 3 weeks. Watchorn & McCance (1937) observed that hyperexcitability did not occur in larger rats. These authors also reported a marked fall in magnesium concentration in bones, teeth and serum. The magnesium content of soft tissues was unaltered except in skin and kidney. In the latter organ a large increase in calcium content occurred. Tufts & Greenberg (1938) found a slight reduction of magnesium in brain and muscle, and an increase of calcium in heart, muscle and kidney. Darrow, Harrison & Taffel (1939) considered that electrolyte analyses were less variable when expressed in terms of dry fat-free solids, a procedure followed in the present experiment and by Cotlove, Holliday, Schwarz & Wallace (1951) but not by previous workers.

In the present experiment little or no significant change in magnesium concentration was detected in brain, liver or kidney, although had the results been expressed in terms of another frame of reference, such as non-collagen nitrogen (Lilienthal, Zierler, Folk, Buka & Riley, 1950), differences might perhaps have been found. In skeletal muscle there was unequivocal evidence of progressive and eventually severe depletion of both magnesium and potassium with a large increase of sodium and chloride. Although potassium depletion is usually associated with an increase in intracellular sodium, this is not invariably found (Holliday & Segar, 1957). Cotlove *et al.* (1951) have described a lesser degree of potassium depletion in rats subjected to milder and less prolonged magnesium deficiency.

It is of interest that depletion of dietary potassium also produces much greater change in potassium content of muscle than of the parenchymatous organs (Darrow & Miller, 1942).

Histological study of the kidneys from the rats in the present experiment suggested that loss of kidney tissue due to nephrocalcinosis was not likely to account for the potassium deficit (Cullen, Davidsson & MacIntyre, 1957, unpublished work) and potassium deficiency still occurred in one of the animals with a normal renal calcium.

Vitale, Nakamura & Hegsted (1957) showed that isolated heart, liver and kidney mitochondria from magnesium-deficient rats were abnormal as judged by the uncoupling of oxidative phosphorylation found under their experimental conditions. Macfarlane & Spencer (1953) showed that rat-liver mitochondria could maintain gradients of sodium and potassium in the presence of an adenine nucleotide and that this ability was linked with their capacity for oxidative phosphorylation and with the prevention of swelling; Bartley & Davies (1954) obtained similar results with sheep kidney. Tapley (1956) has since demonstrated that magnesium protects isolated rat-liver mitochondria from the effects of various agents which cause swelling. Baltscheffsky (1957) found that rat-liver mitochondria swelled within a few minutes of being placed in a magnesium-free reaction medium, and then showed uncoupling of oxidative phosphorylation.

Bartley, Davies & Krebs (1954) point out that mitochondria may be the basic units responsible for active transport in the cell and it is possible that the potassium deficit found in the present experiment was due to an effect of magnesium deficiency on ion transport *in vivo*. A failure from this cause to maintain normal potassium gradients especially, but not necessarily, by the kidney could account for our results.

Fitzgerald & Fourman (1956) attempted to produce dietary magnesium deficiency in man. Two subjects consumed a deficient diet for 20 and 27 days: the serum magnesium did not fall but retention of sodium and chloride occurred. No potassium deficiency was found. It is difficult to draw definite conclusions from this experiment because the diet was deficient in respects other than magnesium concentration, as shown by loss of weight in the control period.

The present findings in muscle bear a resemblance to the effects of primary aldosteronism in man (Conn, 1955). In this condition magnesium and potassium deficiency coexist with sodium retention. Baldwin, Robinson, Zierler & Lilienthal (1952) have noted that magnesium and potassium commonly change together in disease in human muscle, although such a relation was not found in rat muscle when primary potassium deficiency was produced (Cotlove *et al.* 1951).

The changes in bone are as described by Watchorn & McCance (1937). There is some evidence from our results of an absolute decline in the bone magnesium in the early weeks of the deficiency. The production of hypercalcaemia has not previously been recorded in experimental magnesium deficiency although nephrocalcinosis has frequently been detected. In pathological conditions in man nephrocalcinosis is often associated with hypercalcaemia.

Parathyroid hormone is considered to be the main regulator of plasma-calcium level, and although it is possible that hyperparathyroidism was produced in the present experiment no direct evidence of this was obtained.

SUMMARY

1. The effects of dietary-magnesium deficiency have been studied in rats (mean initial weight 100 g.). The animals grew little or lost weight and

after 12 days developed peripheral vasodilatation and oedema which lasted for a further 7–10 days.

2. There was a progressive fall in magnesium content in skeletal muscle to 78% of the control value after 64 days, described by the equation: muscle magnesium (m-equiv./kg. of dry fat-free solids) = 102·6–0·353 (days on deficient diet). The potassium content of skeletal muscle fell to 80% of the initial content after 64 days, was closely correlated with muscle magnesium ($r = 0·93$), and was described by the equation: muscle potassium = 73 + 3·55 (muscle magnesium).

3. The mean contents of muscle sodium and chloride increased in the deficient group by 51 and 35 m-equiv./kg. from the control values of 92 and 60 m-equiv./kg. respectively. Assuming all muscle chloride to be extracellular, intracellular sodium in muscle was calculated and found not to differ in the two groups.

4. Magnesium and potassium concentrations were unaltered in brain, liver and kidney.

5. The calcium contents of muscle, brain and liver did not differ significantly in the two groups but there was a mean increase of 40 m-equiv. of calcium/kg. in the kidneys of the deficient group from the control value of 12 m-equiv./kg.

6. Within 3 weeks there was a decline in magnesium concentration in the femur to about half that of the control group.

7. The plasma magnesium concentration fell from the control value of 1·7 to about 0·4 m-equiv./kg. within 3 weeks and thereafter increased slightly. Plasma sodium was slightly increased in the deficient group; the mean calcium concentration in the deficient and control groups was 6·4 and 5·3 m-equiv./kg. respectively.

8. Nephrocalcinosis occurred only when hypercalcaemia was also present.

9. The hypothesis is advanced that the potassium deficiency was due to impaired ion transport secondary to an effect of magnesium deficiency on mitochondrial function.

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The Estimation of Iodine in Biological Materials: a Modification of the Method of Ellis & Duncan

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Most methods described in recent years for the estimation of iodine in biological materials employ a preliminary step in which organic matter is ashed and iodine is converted into iodide. This may be achieved by an acid wet-ashing technique during which the iodine is oxidized to iodate by chromium trioxide or potassium permanganate; the iodate is treated with phosphorous acid or oxalic acid and the liberated iodine distilled into alkali, forming iodide (Chaney, 1940; Talbot, Butler, Saltzman & Rodriguez, 1944; Taurog & Chaikoff, 1946; Barker, 1948; Connor *et al.* 1949; Kydd, Man & Peters, 1950; Moran, 1952; Ellis & Duncan, 1953; Butler & Johnson, 1957). Alternatively, iodide is liberated from organic material by fusion with alkali and eluted with acid (Barker, Humphrey & Soley, 1951; Brown, Reingold & Samson, 1953; Grossmann & Grossmann, 1955; Acland, 1957). The amount of iodine isolated by these procedures is usually determined by measuring its catalytic effect on the ceric sulphate–arsenious acid reaction, which can be followed spectrophotometrically.

We required a method which could be applied to the analysis of iodine in water, milk and urine, and of protein-bound iodine in serum. The alkaline-incineration technique was discarded because of the large sample volumes. A method employing the Riggs modification of the Chaney still was found to be unsatisfactory in our hands, as the recovery of iodine from iodide in aqueous solution was 80% with a standard deviation of 23. The procedure used was based on the methods of Talbot *et al.* (1944) and Taurog & Chaikoff (1946), and is used in the Veterinary Laboratory of the Ministry of Agriculture and Fisheries, Weybridge, Surrey (private communication). Butler & Johnson (1957) have stressed the importance of the dimensions of the still, and our stills may have been faulty.

The technique described by Ellis & Duncan (1953) also gave very variable results when applied to the analysis of iodide in aqueous solution. After the digestion and distillation procedures, as well as the spectrophotometric estimation of iodide had been modified, a method was developed which permits a more reliable estimation of microgram quantities of iodine in water, milk, urine, serum and other biological materials.

EXPERIMENTAL

General procedure for all samples

Cleaning of apparatus. All glassware is cleaned overnight in chromic acid–sulphuric acid. It is thoroughly washed with tap water and then five times with a special water which has been purified by a Permutit Deioniser (portable Deminrolit plant) and subsequent distillation from an all-glass still. Glassware is dried in a hot-air cabinet at 50°.

Reagents. [Mainly according to Ellis & Duncan (1953) and Lein & Schwartz (1951).] Chromium trioxide solution: British Drug Houses Ltd. laboratory reagent (recrystallized), 50% (w/w) solution in water. Sulphuric acid: A.R., concentrated. Phosphorous acid: 1000 g. of H_3PO_3 (British Drug Houses Ltd. laboratory reagent, crystalline) was boiled with 1·5 l. of water for 2 hr., cooled and diluted to 2 l. with water. Hydrogen peroxide: A.R. 30% (w/v). Arsenious acid solution, 0·1 M: 9·9 g. of As_2O_3 (A.R.) was dissolved in 60 ml. of hot N-NaOH and 200 ml. water was added; the solution was neutralized (pH 7) with sulphuric acid and diluted to 1 l. with water. Sodium chloride reagent: 50 g. of NaCl (A.R.) dissolved in 7 N-H_2SO_4 to give 1 l. of solution. Ceric ammonium sulphate solution, 0·01 M: 6·33 g. of $Ce(SO_4)_2,2(NH_4)_2SO_4,2H_2O$ (low in other rare earths, Harrington Brothers Ltd.) was dissolved in 3·6 N-H_2SO_4 to give 1 l. of solution. Sodium arsenite solution: $NaAsO_2$ (British Drug Houses Ltd. laboratory reagent) was dissolved in water to give 1% (w/v) solution. Standard potassium iodide solution: 1 ml. of solution contained 100 μmg. of iodine. When the iodine content of the combined reagents exceeds 100 μmg., this is usually due mainly to the sulphuric acid or the chromium trioxide. The impure reagents are replaced by sufficiently pure ones.

Apparatus. Digestion is carried out in a 125 ml. Kjeldahl flask, and from this flask the iodine vapour is subsequently distilled. The distillation apparatus (Fig. 1) is based on the Ellis & Duncan (1953) still, with the following modifications:

(*a*) Introduction of the three-way tap (*T*).

(*b*) Introduction of potassium hydroxide and calcium chloride traps.

(*c*) Alterations in the sizes of jets *A*, *B* and *C* to 1·2, 1·6 and 1·2 mm. respectively.

(*d*) Connexion of the Kjeldahl flask and the traps with rubber tubing to allow flexibility.

The flow meters were supplied by Rotameters Ltd., and were calibrated to measure from 100 to 5000 ml. of air/min.

Digestion of sample. Duplicate samples, between 0·5 and 50 ml., are measured into 125 ml. digestion flasks. After addition of 5 ml. of chromium trioxide solution and six glass beads (diam. 4·5 mm.) the mixture is shaken and cooled in ice–water while 30 ml. of concentrated sulphuric acid is added carefully, with constant mixing. A 0–250° thermometer is placed in the flask, which is then heated on a sand bath; the sand is 0·5 cm. deep and heat is supplied by Bunsen burners fitted with flame spreaders operating at maximum intensity with the top of the flame touching the sand tray. When the temperature of the mixture reaches 180° the flask is removed, the contents allowed to cool to 100° and 15 ml. of water is then added. Into the flask is introduced a piece of right-angled glass tubing of internal diameter 0·5 cm. and with the vertical arm drawn out into a jet of internal diameter. 0·2 cm., which extends to the bottom of the flask. The mixture is heated to 200° and then removed from the sand bath. A stream of air from a compressed-air cylinder is blown through the glass tubing into the flask at a pressure of 1–2 lb./in.^{2} for 3 min. to remove volatile interfering substances, mainly SO_2. When the digest has cooled to 100°, the thermometer and glass tubing are washed with water and removed from the flask; water is added until the volume of liquid is about 50 ml.

Distillation. The flask is placed in the water bath at 90° for 10 min. and the sections of the distillation apparatus are connected by means of rubber tubing of 6 mm. internal diam. which has been washed with purified water. Volumes (2 and 1 ml.) of 1% sodium arsenite solution are placed in traps 1 and 2 (see Fig. 1) and air is drawn through the apparatus at a rate of 1600 ml./min., the three-way tap being turned so that all the air first passes through the potassium hydroxide solution. Phosphorous acid (3 ml.) is measured into a 10 ml. beaker and drawn from there into the apparatus by holding the beaker at point *X* (Fig. 1) and turning the tap momentarily in the appropriate direction. The beaker is washed twice with small volumes of water and the washings are drawn into the digest. Hydrogen peroxide (0·2 ml.) is added in the same way. After 5 min., another 3 ml. portion of phosphorous acid is mixed with the liquid in the flask, and this is repeated 5 min. later. After a further 5 min. the air flow is stopped, the two traps are detached from the still and their contents quantitatively transferred to a 25 ml. graduated cylinder, the volume being made up to 20 ml. with purified water.

Spectrophotometric estimation of iodine. Sodium chloride reagent (2 ml.) and 0·1 M-arsenious acid (2 ml.) are measured into 15 cm. × 1·5 cm. Pyrex test tubes. Duplicate 5 ml. portions of the distillates are added and the total volume in each tube is made up to 10 ml. with water. All the tubes are covered with a piece of Parafilm (Gallenkamp and Co. Ltd., Sun Street, London, E.C. 2), and shaken well and placed in a water bath at room temperature, which usually lies between 15° and 26°. The bath is placed in a draught-free position so that the temperature of the water does not vary by more than ±0·2° over a period of an hour. After 15 min. has been allowed for equilibration, 1 ml. of 0·01 M-ceric ammonium sulphate solution is added to the first tube and, at 45 sec. intervals, to the remaining tubes, each one being shaken thoroughly before being replaced in the bath. After a suitable time interval, which varies from 20 min. at 26° to 40 min. at 15°, some of the solution from the first tube is transferred to a 1 cm. glass cuvette, and the extinction ($E_{1\text{ cm.}}$) measured at 363 mμ in a Unicam spectrophotometer (SP 600). The amount of iodine present in the distillate portion is calculated from a calibration curve prepared as described below, which relates log $E_{1\text{ cm.}}$ to iodide concentration. The total amount of iodine in both the sample and the reagents together is found by multiplying this value by four.

Reagent blank. Purified water equal in volume to the sample is analysed in duplicate as described above, and the amount of iodine present in the reagents is calculated. This amount is subtracted from the value for sample and reagents to give the iodine content of the sample.

Calibration curve. Volumes (0·25, 0·50, 0·75 and 1·00 ml. respectively) of the standard potassium iodide solution are added to tubes containing sodium chloride reagent (2 ml.) and arsenious acid (2 ml.); 0·75 ml. of sodium arsenite solution is measured into each tube to equal the amount of this reagent in 5 ml. of distillate. A fifth tube containing no added iodide is prepared in a similar manner. The total volume of all five tubes is made up to 10 ml. with purified water and the spectrophotometric analysis carried out as described above. The plot of the logarithm of the extinction against the amount of iodine added as iodide to each tube is linear.

A calibration curve is prepared for each set of estimations, and the five tubes containing the standard iodide solution are mixed randomly with those prepared from the samples.

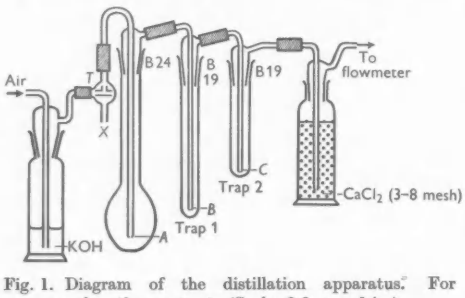

Fig. 1. Diagram of the distillation apparatus. For explanation see text. (Scale: 3·2 mm.: 1 in.)

Iodine estimation in various materials

Natural waters. A volume (50 ml.) of the water is measured in a digestion flask, and the digestion reagents are added. The analysis is carried out as described above.

Milk. Approx. 2 g. of milk is added to a 10 ml. beaker, and the exact weight is determined. The milk is then transferred to a digestion flask together with 20 ml. of water. The iodine content is estimated by the general procedure.

Urine. The human urine is collected over a 24 hr. period, in a 2·5 l. bottle containing 20 ml. of toluene. The volume of urine analysed by the general procedure varies from 2 to 8 ml., depending on the intake of iodine.

Protein-bound fraction in serum. A volume (3 ml.) of serum is mixed with 20 ml. of water in 70 ml. polythene bottles fitted with screw caps, and 3 ml. of 10% (w/v) sodium tungstate and 3 ml. of 0·67 N-sulphuric acid are added (Moran, 1952). The bottle is completely filled with water before centrifuging to prevent collapse at the neck,

and is then spun at 5500 *g* in a BaraGyro centrifuge for 20 min. The supernatant is decanted and the residue suspended in water by shaking vigorously. A volume (3 ml.) of 0·67 N-sulphuric acid is added, and then 3 ml. of 10% sodium tungstate solution, and the contents of the bottle are well mixed. The bottle is filled with water and centrifuged as before. The procedure is repeated once more, and the washed precipitate transferred with 20–25 ml. of water to a digestion flask.

Recovery experiments

Recovery of ^{131}I. In all radioactivity measurements, 10 ml. of solution was counted in a Twentieth Century Electronics liquid counter, type M6H. Counts were corrected for background count, and were accurate to ±2% unless otherwise stated. Radioactive iodide was obtained in the form of tracer ^{131}I in dilute sodium thiosulphate solution at pH 8–10 (Radiochemical Centre, Amersham, Bucks). The tracer iodine was diluted with potassium iodide solution until the total concentration of iodine was approx. 100 μmg./ml., and the radioactive count was approx. 2000 counts/min./ml. A volume (2 ml.) of this solution was used to determine the recovery of iodine from small volumes of an aqueous solution of potassium iodide, and from volumes of urine (sample A) varying from 2 to 10 ml.

Recovery of iodine from potassium iodide. (1) *In aqueous solution.* Volumes of potassium iodide solution varying from 0·5 to 5 ml. were added to digestion flasks, and the sides of the flasks were washed down with small volumes of water. The iodine contents of the samples were determined, and the percentage recovery of iodine was calculated.

To determine the recovery of iodine under the conditions used in the analysis of natural waters, 2 ml. of the standard iodide solution was diluted to 50 ml. before digestion.

(2) *In milk.* The iodine content of milk (sample A) was determined in the way described above; 2 ml. of standard iodide solution was added to ten samples of milk A and the mixtures were analysed. The iodine content of milk (sample B) was estimated and 0·5 ml. of standard iodide solution was added to each of five samples of this milk.

(3) *In urine.* Samples (3 ml.) of a urine (sample B) were analysed as described above. A volume (2 ml.) of the standard iodide solution was used to determine the percentage recovery of iodine from urine in the presence of toluene.

Recovery of protein-bound ^{131}I. ^{131}I-radio-iodinated human-serum albumin (0·24 ml.), containing 4·8 mg. of albumin (Radiochemical Centre, Amersham, Bucks.) was diluted with bullock serum until 3 ml. of the diluted material gave a count of approx. 2000 counts/min. The radio-iodinated albumin contained not more than 2% of free iodine, and a correction was applied for this amount. The serum was treated as described in this paper, and the recovery of the bound iodine was calculated. The effect on the recovery when tungstic acid was omitted in the washing procedure was determined.

Two solutions, A and B, containing known amounts of ^{131}I were prepared by diluting tracer ^{131}I in sodium thiosulphate solution with potassium iodide solution. In solution A 0·2 ml. contained about 30 μmg. of iodine, and 0·2 ml. of solution B contained about 330 μmg. of iodine. Four polythene bottles, each containing 0·2 ml. of solution A and 3 ml. of bullock serum, were incubated for 2 hr. at 37°. The protein-bound iodine was precipitated and washed in the usual way, and the supernatant, washings and washed precipitate were all collected, and their radioactivity was determined as a percentage of the amount added. This was repeated by using 0·2 ml. of solution B, and incubating for 16 hr. with 3 ml. of bullock serum before precipitating the protein.

RESULTS AND DISCUSSION

Comparison of the present method with that of Ellis & Duncan

Digestion. The digestion stage of chromic acid-ashing methods for estimating iodine was regarded by Moran (1952) as being the most critical part of the analysis. He suggested that excessive heating results in loss of iodine, probably due to partial reduction of chromic compounds and of iodate, and that insufficient heating does not expel chlorides, which interfere with the catalytic action of iodine on the ceric sulphate–arsenious acid reaction. Ellis & Duncan (1953) reported that if only one portion of water is boiled off in the digestion, the passage of air through the digest for 10 sec. periods at 1–2 min. intervals is necessary in order to remove substances which would cause inaccuracies in the spectrophotometric estimation of iodine. In this Laboratory, passing a current of air through the liquid for 3 min. after digestion has been completed has been found to be equally satisfactory in removing these interfering substances.

When the end points of the two stages of the digestion were determined according to the method of Ellis & Duncan (1953) the mean recovery of radioactive iodine from radioactive Na^{131}I was 86%, S.D. 12·6% (five estimations). With the method described in this paper, the recovery of ^{131}I was 94%, S.D. 2·2% (four estimations). Calculation of the variance ratio showed that there was a significant decrease in accuracy when the procedure of Ellis & Duncan (1953) was adopted ($P = 0{\cdot}01$–$0{\cdot}001$).

If the temperature at the end of the first heating in the digestion was raised from 180° to 200°, the recovery of radioactive iodine was unaffected, and this was also true if the digest was heated to 220° in the second stage of the oxidation process.

Distillation. The estimation of iodine by the use of a still designed by Chaney (1940) and a chromic acid-ashing method was investigated by Moran (1952), who found that the ratio of the weight of phosphorous acid added in the distillation to the weight of chromium trioxide used in the digestion is an important factor in obtaining the maximum recovery of iodine, the optimum ratio being 3 g. of phosphorous acid to 2 g. of chromium trioxide.

In the method of Ellis & Duncan (1953), the ratio of the weights of phosphorous acid to chromium trioxide is 1:3·2. We found it convenient to use 4 g. of chromium trioxide rather than 3·2 g.

because in milk analyses 2 g. of a high-fat milk reduces more than 3 g. of chromium trioxide. The addition of 1·25 g. of phosphorous acid in the distillation gives approximately the same ratios of the reagents to each other as those quoted by Ellis & Duncan (1953); this amount of phosphorous acid is contained in 2·5 ml. of the 50 % phosphorous acid (Table 1, group *a*). Chaney (1940) found that with some bottles of phosphorous acid the recovery of iodine is inexplicably low, but that the addition of 2 drops of 30 % (w/v) hydrogen peroxide improves the recovery (cf. Moran, 1952 and Table 1, group *b*). Taurog & Chaikoff (1946) did not use hydrogen peroxide in their method of estimating iodine as they found that it frequently caused a green colour to appear in the distillate.

Moran (1952) reported that the recovery of iodine increases with the length of the distillation time, and concluded that the reduction of iodate does not take place immediately on the addition of the reducing agent, possibly due to the presence of a complex between iodate and chromic acid. For this reason phosphorous acid was added in several portions (Table 1, groups *e*, *f*, *g*).

In order to test the efficiency of the distillation under different conditions, known amounts of $Na^{131}I$ were ashed and the percentage of ^{131}I recovered in the distillate was calculated (see Table 1). It is shown by Student's *t* test after using an angular transformation that there was no significant difference in the mean percentage recoveries of groups *b* to *g* inclusive, but that the addition of hydrogen peroxide in the distillation causes a significant increase in the percentage recovery of ^{131}I ($P < 0{\cdot}001$). There was a significant decrease in variance when 6 ml. of phosphorous acid was added in two fractions at intervals of 5 min. rather than as one portion at the beginning of the distillation ($P = 0{\cdot}05$–$0{\cdot}02$). Omission of the arsenious acid affected neither the mean percentage recovery nor the variance. Arsenious acid has therefore been omitted in the final method.

With the treatment described in group *g* (Table 1) and sodium arsenite as the trap solution the mean recovery of 100–400 μmg. of iodine added as potassium iodide was 90·3 % (29 estimations); with the 200 μmg. amount, the S.E. of a single observation was 5·0 %. At the point in the estimation at which the distillation was usually terminated, a further 3 ml. of phosphorous acid was added to the flask and distillation continued for a further 5 min. Eleven determinations were thus carried out in which the samples for analysis were 200 μmg. quantities of iodine as potassium iodide, and 12 determinations were made of 50 μmg. amounts of iodine. The mean recovery of 200 μmg. of iodine was 98 %, S.E.M. 1·9, and that of 50 μmg. of iodine was 102 %, S.E.M. 5·8. The increases in the mean percentage recoveries of 200 and 50 μmg. of iodine were significant ($P = 0{\cdot}02$–$0{\cdot}01$ and $P = 0{\cdot}01$–$0{\cdot}001$ respectively) but there was no significant increase in reproducibility.

The method of distillation shown in Table 1, group *g*, was adopted as the usual procedure, after considering the accuracy of the technique, the mean percentage recovery of added iodide and the time taken to carry out the estimation.

Trapping of distilled iodine. The composition of the solution which traps the distilled iodine in methods using chromic acid and phosphorous acid has varied considerably. Sodium hydroxide was found to be satisfactory by Taurog & Chaikoff (1946) and Moran (1952). In his early experiments, Barker (1948) used sodium hydroxide to trap the iodine and obtained very low recoveries when the iodine in the distillate was estimated spectrophotometrically, but on replacing the sodium hydroxide by sodium sulphite the recovery was increased to 90 %. In this procedure the sulphite had to be removed before the colorimetric estimation, as otherwise it caused immediate fading of the ceric ammonium sulphate. Ellis & Duncan (1953) trapped the iodine with a mixture of sodium hydroxide and sodium arsenite; the arsenite did not interfere with the spectrophotometric estimation.

By the method described in this paper, except that the trap solutions were replaced by those of

Table 1. *Effects of varying the types and quantities of reagents added during the distillation*

Four analyses were carried out for each group of results. Portions (2 ml.) of a solution of $Na^{131}I$ containing approx. 200 μmg. of iodine were treated as described in the text and distilled under the conditions specified in the table. The amount of ^{131}I recovered in the distillate was determined as described in the text. The arsenious acid solution contained 0·013 g. of arsenic trioxide/ml. in 2·5 N-sulphuric acid and was added in three 1 ml. portions at the beginning of the distillation. Hydrogen peroxide was added at the beginning of the distillation. When more than one portion of phosphorous acid was added, the first addition was made at the commencement of the distillation, and subsequent additions were made at 5 min. intervals. Distillation was continued for 15 min. after the addition of the first batch of reagents. The solution used to trap the iodine was a mixture of sodium hydroxide and sodium arsenite (Ellis & Duncan, 1953).

	Volume of reagents added during distillation (ml.)			Mean recovery	
Group	H_3PO_3	As_2O_3	H_2O_2	(%)	S.D.
a	2·5	3	—	52	16·8
b	2·5	3	0·2	87	6·3
c	2·5	3	0·5	87	8·7
d	6·0	3	0·2	89	8·3
e	2 × 3	3	0·2	95	2·6
f	3 × 3	3	0·2	96	1·3
g	3 × 3	—	0·2	93	2·3

Ellis & Duncan (1953), the mean percentage recovery of radioactive iodine from $Na^{131}I$ was 92 %, S.D. 5 % (14 estimations). Under the same conditions the apparent recovery of iodine from potassium iodide, determined with the spectrophotometric procedure as the final stage of the analysis, was very variable, frequently being of the order of only 50 %. The iodine was therefore being carried into the trap, but either (i) the iodine was present in a non-catalytic form, such as iodate, or (ii) some substance was interfering with the catalytic action of the iodine. Iodine distilled over into sodium hydroxide in the form of iodine or hypoiodous acid gives rise to iodate.

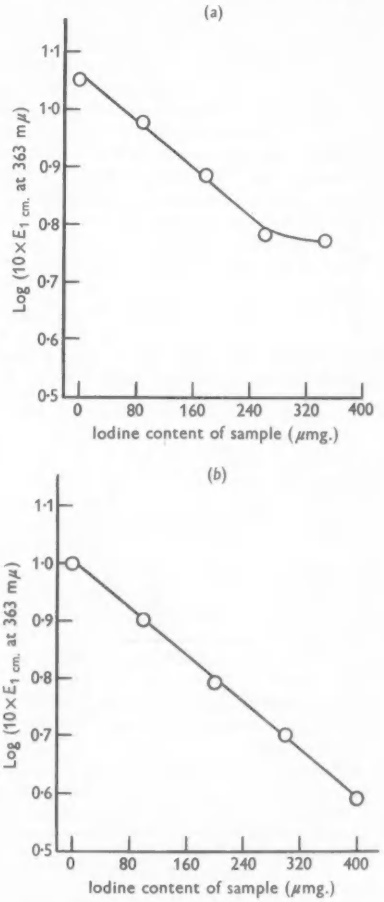

Fig. 2. Effect of the volume of 1 % sodium arsenite solution in the still traps on the relation between the amount of iodine added to the reagents before analysis and the extinction at 363 mμ at the end of the spectrophotometric determination of iodide. (*a*) Volumes of potassium iodate solution containing 88–346 μmg. of iodine were analysed as described in the text, except that the still traps contained 1·5 and 0·5 ml. of 1 % sodium arsenite solution respectively. (*b*) Volumes of potassium iodide solution containing 100–400 μmg. of iodine were analysed as described in the text, the volume of 1 % sodium arsenite solution in the traps being 2 and 1 ml. respectively.

Ellis & Duncan (1953) added 1 ml. of 1 % (w/v) sodium hydroxide and 3 drops of 0·1 N-sodium arsenite to trap 1 and 0·5 ml. of 1 % sodium hydroxide to trap 2. These were changed to 1·5 ml. of 1 % (w/v) sodium arsenite in trap 1 and 0·5 ml. of 1 % sodium arsenite in trap 2. Recoveries of iodine estimated spectrophotometrically averaged 87 % over the linear portion of the curve but fell when the sample iodine exceeded 280 μmg. (Fig. 2*a*). Increasing the volume of arsenite to 2 ml. in trap 1 and 1 ml. in trap 2 increased the recovery of iodine to 89 % in the higher range (Fig. 2*b*).

The importance of the rate of air flow through the still was stressed by Ellis & Duncan (1953), who reported that the use of an air flow lower than 1500 ml./min. results in incomplete transfer of iodine into the traps, whereas when this rate is exceeded, the distillate is coloured green owing to the transference of chromium ions. With the jet sizes quoted in their paper, experiments showed that even at an air flow of 1500 ml./min. some of the distillates were pale green in colour. In the Ellis & Duncan (1953) still, the tips of the jets increased in size in the direction in which the air passed through the still. A slight vacuum was therefore formed in the Kjeldahl flasks during the distillation and this appeared to facilitate the transfer of chromium ions as well as of iodine into the traps. It was found that if the jets were made approximately equal in diameter, thus avoiding the vacuum, the flow could be raised to over 2000 ml./min. without a green colour appearing in the distillate or the percentage recovery of iodine from iodide altering significantly.

Spectrophotometric determination of iodide

In most methods in which the reduction of Ce^{4+} ions by arsenious acid has been followed in the visible part of the spectrum, the wavelength at which measurements have been made has been 415 or 420 mμ. The absorption curve of ceric ammonium sulphate solution is shown in Fig. 3. Because the absorption of this solution at 363 mμ is about nine times greater than at 420 mμ the former wavelength was used. Although there is a maximum in the absorption curve at 315 mμ (Weybrew, Matrone & Banley, 1948; Freedman &

Hume, 1950), measurements could not be carried out at wavelengths below 363 mμ owing to limits imposed by the spectrophotometer.

The effect of the concentration of I^- ions on the rate of the reduction of Ce^{4+} ions by arsenious acid is shown in Fig. 4. The reaction can be followed satisfactorily at room temperature and the time required for the extinction to change by a suitable amount varied from 40 min. at 15° to 20 min. at 26°.

In the spectrophotometric analysis of solutions containing known amounts of iodine, the standard error of a single result was 1·4 μmg., for amounts of iodine between 25 and 100 μmg. (48 results).

Estimation of iodine in the presence of other compounds

It is more convenient to prepare a calibration curve by adding iodide to water and sodium arsenite, rather than to the distillate from a reagent blank. This practice assumes that no compound which distils from the reagents into the traps has any effect on the catalytic action of iodine on the ceric–arsenite reaction.

To determine if this assumption is correct, twenty-five tubes containing known amounts of standard iodide solutions were analysed spectrophotometrically, and the log extinction values at the end of the reaction time were calculated. At the same time nineteen portions of the distillates from reagent blank analyses were mixed with known amounts of iodide solution, and treated in a similar manner. The regression coefficients of the log extinction, on the amount of iodide added, were calculated.

The experiment was completed by the analysis of duplicate samples of 500 mg. of sodium chloride and of 500 mg. of sodium bromide. The distillates from these analyses were divided into portions and to each portion a known amount of iodide was added. After spectrophotometric analysis of these solutions, the regression coefficients of log E, on the amount of iodide added, were calculated (Table 2).

The values of the regression coefficients showed that there was no interference with the catalytic action of iodine due to any compounds distilled from the reagents, and that even the presence of very large quantities of chloride and bromide in the sample analysed did not affect the rate at which the ceric–arsenite reaction was catalysed by the iodine.

The analyses did not show any difference between the iodine content of the reagents, and of

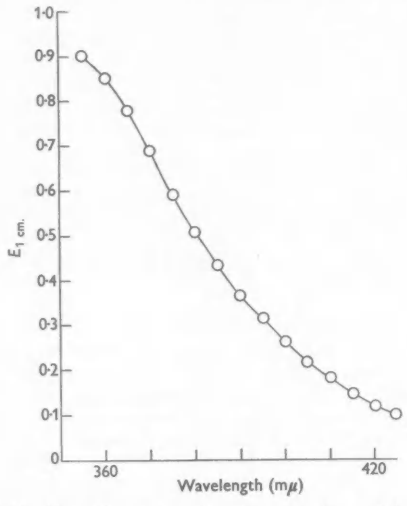

Fig. 3. Absorption curve of ceric ammonium sulphate. The absorption of a 0·3 mM-solution of ceric ammonium sulphate was measured in a Unicam spectrophotometer model SP. 600, in 1 cm. glass cuvettes.

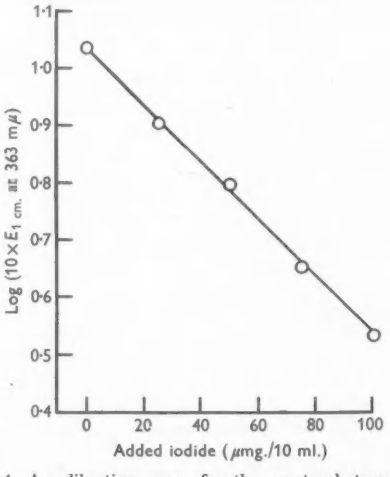

Fig. 4. A calibration curve for the spectrophotometric estimation of iodide. Volumes (0·25, 0·50, 0·75 and 1·00 ml.) of standard potassium iodide solution were added to 2 ml. of sodium chloride reagent (100 mg. of NaCl in 7 N-H_2SO_4), 2 ml. of 0·1 M-arsenious acid and 0·75 ml. of 1% sodium arsenite solution. Water was added to give a total volume of 10 ml., and the tubes were placed in a water bath at room temperature (15·1°) for 15 min. A volume (1 ml.) of 0·01 M-ceric ammonium sulphate solution was added to each solution, 45 sec. elapsing between successive additions. After a reaction time of 37 min. the extinction $E_{1\,cm.}$ was measured at 363 mμ in a Unicam spectrophotometer model SP. 600.

Table 2. *Effect of compounds other than iodides on the catalytic action of iodine on the ceric ammonium sulphate–arsenious acid reaction*

The reagents, and the reagents with added chloride or bromide, were digested, and the iodine was distilled as described in the text. Volumes of standard iodide solution from 0·25 to 1·0 ml. were added to portions of the distillates and the solutions analysed spectrophotometrically.

Solution to which KI was added	$10^3 \times$ regression coefficient (log E/μmg. of I added)	No. of points from which regression coefficient was calculated
Water (calibration curve)	5·589	25
Distillate from reagent blank	5·597	19
Distillate from analysis of 500 mg. of NaBr	5·639	8
Distillate from analysis of 500 mg. of NaCl	5·671	8

the reagents with added chloride or bromide. In order to ensure that the presence of chloride did not cause the loss of any iodine from the sample being analysed, the recovery of iodine from potassium iodide containing 200 μmg. of iodine in the presence of 500 mg. of sodium chloride was determined and found to be 94 %.

If the spectrophotometric determination is carried out with the omission of the sodium chloride reagent, the presence of large amounts of chloride in the sample analysed results in interference with the catalytic action of the distilled iodine on the reduction of Ce^{4+} ions by arsenious acid. If the sample contains a large amount of bromide, this effect is not observed. The addition of Cl^- ions in the spectrophotometric analysis of iodide has been recommended by several workers (Barker, 1948; Connor *et al.* 1949; Carr, Graham, Ober & Riggs, 1950; Lein & Schwartz, 1951). Lein & Schwartz (1951) have suggested that this addition of a large excess of Cl^- ions prevents the combination of I^- ions with undefined contaminants. These contaminants would otherwise have interfered with the catalytic action of the iodide. It is possible that the reactions between chloride, sulphuric acid and chromium trioxide lead to the subsequent distillation of similar contaminants; when bromide is substituted for chloride these contaminants do not seem to be formed.

Fluoride ions at concentrations less than 50 μg./10 ml. do not interfere with the spectrophotometric determination of iodide. At larger concentrations of fluoride the catalytic action of iodide is partially inhibited.

Recovery experiments

Aqueous potassium iodide solution. The recovery of quantities of iodine between 50 and 400 μmg. added in the form of potassium iodide in from 0·5 to 4 ml. of solution was determined by the present method (Table 3).

There was no significant difference between the variance values for the different amounts of iodine, but there was a significant difference in the recovery of 50 μmg. of iodine compared with that of 100 μmg. of iodine ($P = 0{\cdot}02$–$0{\cdot}01$). The standard error of a single estimation was 10·1 μmg. of iodine, and the mean percentage recovery of quantities between 100 and 400 μmg. of iodine inclusive was 90·3 %.

Table 3. *Recovery of iodine from small volumes (0·5–4 ml.) of an aqueous solution of potassium iodide*

Volumes (0·5, 1·0, 2·0, 3·0 and 4·0 ml.) of standard potassium iodide solution were analysed by the present method.

Iodine added (μmg.)	No. of estimations	Mean recovery (%)	S.D.
50	10	72	14
100	14	90	11
200	6	90	4
300	5	93	5
400	4	88	2

Table 4. *Recovery of iodine from milk samples*

The iodine content of weighed portions of milk (1·74–3·329 g.) was determined as described in the text. Standard potassium iodide solution (2 or 0·5 ml.) was added to further weighed portions of the same milk and the recovery of the added iodine was calculated.

Milk sample	No. of determinations	Mean wt. of milk analysed (x g.)	Iodine added to x g. of milk (μmg.)	Mean total iodine in x g. of milk (μmg.)	Variance	Mean recovery (%)	Mean total iodine in milk sample ± S.E.M. (μg./kg.)
A	10	1·82	—	53	22·9	—	28·9 ± 0·8
A	10	1·82	200	244	87·3	95·3	—
B	5	2·62	—	24	92·0	—	9·3 ± 1·4
B	5	2·37	50	70	23·3	96·8	—

The low concentration of iodine in natural waters necessitated the use of 50 ml. samples in order to obtain an amount of iodine within the range of the method. Experiments were carried out to determine if the use of these large volumes interfered with the recovery of iodine. The mean recovery of 200 μmg. of iodine was found to be 90 %, S.D. 6·2 % (eight estimations). It was concluded that the large volume of water taken did not affect the recovery of iodine from iodide in aqueous solution.

Milk. Samples of two milks, A and B, were analysed to determine their iodine content, and then known amounts of iodine were added as potassium iodide, and the determinations were repeated (Table 4).

There was only a small difference between the recovery of 50 and 200 μmg. quantities of iodine, and no significant difference between the variance values for amounts of iodine between 24 and 244 μmg. The lower limit for the estimation of iodine in milk is of the order of 50 μmg. of iodine, compared with 100 μmg. in waters.

Urine. Stanbury *et al.* (1954), using the modification of the method of Barker (1948) described by Carr *et al.* (1950), found that the recovery of radioactive iodine from urine decreases as the volume of urine taken for analysis increases. In order to determine whether this is true for the method described in this paper, the following experiment was carried out. Volumes of urine measuring between 2 and 10 ml. (urine A) were pipetted into digestion flasks and the sides of each flask washed down with water. Radioactive iodine was added to each sample and the results of the analyses are shown in Table 5.

The mean percentage recovery increased when the volume of urine used was increased from 8 to 10 ml., and this increase was significant ($t = 3·1$, $P = 0·02$–$0·01$). For volumes between 2 and 8 ml. there was no change in the percentage recovery of iodine.

The effect of toluene in the urine on the analysis of iodine was tested in samples of urine B before and after the addition of known amounts of iodine as iodide. The iodine content of 3 ml. of urine was found to be 106 μmg., S.E.M. 2·9 (nine estimations). The 95 % confidence limits for the true mean recovery of 200 μmg. of added iodine lay between 93 and 102 %. Thus the recovery of iodine from urine is not affected by the presence of toluene in the sample. The coefficient of variation for the determination of iodine in urine, calculated from the data for urine B, was 8·2 %.

The mean recovery of iodine from volumes of urine between 2 and 8 ml. was 89·8 % (Table 5).

Serum-protein-bound iodine. Volumes (3 ml.) of ^{131}I radio-iodinated serum were analysed by the procedure described for estimation of protein-bound iodine. The mean percentage recovery of protein-bound ^{131}I was 85 %, S.D. 2·6 (eleven estimations). In a series of analyses in which the addition of sodium tungstate and sulphuric acid was omitted during the washing of the precipitate the mean recovery was 66 %, S.D. 3·3 % (six estimations).

In order to test whether non-protein-bound iodide can become occluded with the precipitate, especially when the level of iodide is abnormally high, a series of experiments were carried out in which radioactive iodide was incubated with serum before precipitation. The division of the iodide between the supernatant, washings and washed

Table 5. *Recovery of ^{131}I from different volumes of urine*

Portions (2 ml.) of Na^{131}I solution containing approx. 200 μmg. of iodine were added to samples of urine (2–10 ml.). Recovery of ^{131}I was determined as described in the text.

Volume of urine (ml.)	Percentage recoveries		Mean percentage recovery
2	88·3	93·2	90·7
4	88·5	88·7	88·6
6	88·5	91·5	90·0
8	91·1	88·5	89·8
10	92·7	96·7	94·7

Table 6. *Recovery of ^{131}I added as inorganic iodide to serum before precipitation of protein-bound iodine*

A volume (0·2 ml.) of a solution of Na^{131}I containing approx. 33 μmg. of iodine was added to 3 ml. of bullock serum and the mixture incubated for 2 hr. at 37°. The protein-bound iodine was precipitated and washed and the amount of ^{131}I in the supernatant and washings was determined as described in the text. The washed precipitate was dissolved in sodium tungstate solution and the radioactivity of the solution determined. This was repeated with 0·2 ml. of Na^{131}I solution containing approx. 330 μmg. of iodine. Radioactivity in the supernatant was determined to an accuracy of 2 %, that in the first washing to 7 % and that in subsequent washings and the residue to 10 %.

Approx. amount of I added (μmg.)	No. of estimations	Mean recovery of ^{131}I (% of total)				
		Supernatant	Wash 1	Wash 2	Wash 3	Residue
33	4	91	6	0	0	0
330	8	92	9	2	1	1

precipitate was determined (Table 6). It was concluded that only two washings were necessary even if the level of inorganic iodine in the serum was abnormally high.

Some other aspects of the method. Alkaline-incineration techniques appear to be satisfactory only when protein is present in the sample being analysed (Acland, 1957). A similar effect of protein has also been observed in some acid-ashing methods (Taurog & Chaikoff, 1946; Barker, 1948). In the present method the recovery of iodine is satisfactory in the presence or absence of protein, and no protein need be added to the sample. Acland (1957) has pointed out that one disadvantage of some acid-ashing processes is the relatively low recovery of the iodine present in the sample. In our method the recovery of iodine from aqueous potassium iodide solutions can be increased from 90 to 98% if desired by extending the distillation time and adding more phosphorous acid. Wet acid ashing does, however, require more constant attention than alkaline-incineration methods, but it can be applied to a wider range of materials. One further disadvantage is the relatively high iodine content of the blank, mainly due to iodine in the sulphuric acid and chromium trioxide. Matthews, Curtis & Brode (1938) have recommended boiling the acid with concentrated hydrochloric acid to remove the iodine, but we have not found this to be satisfactory.

SUMMARY

1. The method of Ellis & Duncan (1953) for the estimation of protein-bound iodine in serum has been extensively modified and applied to the estimation of iodine in natural waters, milk and urine.

2. Factors which were found to be relevant were the temperatures during digestion, the quantities and types of the reagents added during distillation and the nature of the reagents used for trapping the distilled iodine.

3. The iodide obtained on distillation was determined by a modification of the method of Lein & Schwarz (1951), which rests on the catalytic action of iodide on the reduction of Ce^{4+} ions by arsenious acid. The reduction was measured spectrophotometrically at 363 mμ and the reaction was carried out at room temperature.

4. Chloride and bromide were without effect on the estimation of iodine, even when the ratio of chlorine or bromine to iodine exceeds $1{\cdot}5 \times 10^6$ to 1. Concentrations less than 50 μg. of fluoride/10 ml. did not interfere with the spectrophotometric estimation of iodide.

5. The recovery of iodine, added as iodide, from an aqueous solution of potassium iodide (200 μmg. of iodide in 50 ml. of solution) was 90%, S.D. 6·2 (eight estimations). Five determinations on a sample of cow's milk gave a mean of 9·3 μg. of iodine/kg. of milk, S.D. $\pm$ 3·7; ten determinations on another sample of milk gave a mean of 28·9 μg. of iodine/kg. of milk, S.D. 2·6. The mean recovery of iodine from milk samples was 96% (sixteen estimations). The mean iodine content in a 3 ml. sample of human urine was 106 μmg., S.E.M. 2·9 (nine estimations), and the mean percentage recovery of iodine from urine was 89·8% (eight estimations). The mean recovery of ^{131}I-radio-iodinated protein-bound iodine from serum was 85% (eleven estimations).

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Aminopterin Inhibition in *Aerobacter aerogenes*: Alanine and Valine Accumulation During the Inhibition and their Utilization on Recovery

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The inactivation of the folic acid antagonist aminopterin (4-amino-4-deoxypteroylglutamic acid) in cultures of *Aerobacter aerogenes* has been shown to occur by oxidative cleavage with the formation of derivatives of 6-carboxy- and 6-hydroxymethylpteridine (Webb, 1955). Thus during the period of inhibition of growth which follows the addition of bacteriostatic amounts of aminopterin to cultures of *A. aerogenes* in the early logarithmic phase of multiplication, there is a continual decrease in the concentration of the antagonist. Ultimately, this concentration falls to a non-inhibitory level and growth recommences. The growth rate of *A. aerogenes* in a simple chemically defined medium during this recovery phase frequently exceeds that of cultures grown in the absence of the antagonist, and it has been suggested that this is due to the utilization, on the resumption of growth, of metabolites which accumulate throughout the inhibition. Two of these intermediates have now been identified as alanine and valine, and this paper is concerned with the utilization of these amino acids during the recovery from inhibition by aminopterin. It is shown that both amino acids are utilized in the synthesis of leucine, the α- and carboxyl-carbons of which are derived from the accumulated alanine, and the remaining four carbon atoms from the valine. Evidence is presented which indicates that the carboxyl carbon of valine is eliminated, not as carbon dioxide, but as 'active' formate, which is utilized in the synthesis of purine nucleotides and the methyl group of thymine. It is suggested that the production of the C_2 and C_4 intermediates necessary for biosynthesis of leucine from pyruvic acid and 3-methyl-2-oxobutanoic acid is dependent upon folic acid and is inhibited by aminopterin.

MATERIALS AND METHODS

Source of organisms and preparation of cultures. Aerobacter aerogenes and *A. cloacae*, initially obtained from the National Collection of Type Cultures (strain numbers 418 and 408 respectively), were available from the Laboratory collection. *Escherichia coli* (American strain B) was provided by Dr W. J. Nickerson, Institute of Microbiology, Rutgers University, New Brunswick, N.J., U.S.A. The strains were maintained on nutrient-agar slopes and transferred at approximately monthly intervals. Before being used in the following experimental work, the bacteria were serially subcultured in the simple defined medium of Monod (1942), which contained glucose as the sole organic component and was sterilized by filtration through sintered-glass funnels (UF porosity).

Cultures were grown at 37° in 18 ml. test tubes, 250 and 500 ml. Erlenmeyer flasks or 5 l. bolt-head flasks, containing 5, 50, 100 and 1000 ml. of medium respectively, according to the design of the experiment. When growth curves were plotted, measurements of turbidity were made every 15 min. with a Spekker absorptiometer (H. 508 filters), wooden mounts being used to hold the test tubes in the cell compartment of the instrument. To obtain uniform initial turbidites in these experiments a flask of medium (30 ml.) at 37° was first inoculated with 2 ml. of an 18 hr. culture, and portions (5 ml.) of this were transferred to sterile, matched test tubes towards the end of the lag phase. As the tubes were shaken before each determination the frequency of these measurements ensured the regular distribution of the cells and gave reproducible growth curves. In other experiments, in which the larger vessels were used, the cultures were either allowed to remain static or were agitated at intervals with a magnetic stirrer operated at low speed. Aeration was not employed since in media containing aminopterin this caused the rapid inactivation of the antagonist (Webb, 1955). Cell dry weights were obtained from the turbidity readings by means of a calibration curve prepared from cells harvested after growth for 18 hr. in the defined medium.

The final aminopterin concentration incorporated into the growth medium was usually 1:12 000 ($\equiv$0·19 mM). With this relatively high concentration the period of inhibition lasted for 15–18 hr., and thus it was possible to add the antagonist in the late afternoon and follow the subsequent recovery the next morning. The antagonist was added as a sterile (filtered) 1:2000 solution in Monod's medium to cultures in the early logarithmic phase of multiplication (turbidity about 0·15; $\equiv$65 μg. of dry cells/ml.).

In a series of experiments in which a number of compounds were examined for their ability to prevent inhibition by aminopterin when incorporated into the growth medium, the antagonist concentration was reduced to 1:25 000. At this concentration growth curves obtained over a period of 8 hr. were indistinguishable from those obtained at the higher level, although recovery usually occurred within 12 hr.

Paper chromatography. Paper chromatograms were developed by the descending method unless otherwise stated. The following solvent systems were used: (1) Amyl alcohol–methanol–0·2M-sodium citrate buffer, pH 5·0, on paper previously buffered with aq. sodium citrate sol. (J. A. Grunau, unpublished work); (2) butanol–acetic acid–water, 4:1:5 (Partridge, 1948); (3) methanol–aq. 1% (w/v) urea, 95:5 (ascending); (4) *tert.*-amyl alcohol saturated with water; (5) phenol–*m*-cresol–0·2M-borate buffer, pH 9·3, on buffered (borate, pH 9·3) paper (Levy & Chung, 1953); (6) butanol–5N-acetic acid, 7:3 (ascending) (Good & Johnson, 1949); (7) butanol–N-hydrochloric acid, 7:1 (Marshak & Vogel, 1951); (8) water-saturated butanol; (9) butanol–diethyleneglycol–water, 4:1:1 (Vischer & Chargaff, 1948); (10) butanol–2-methoxyethanol–0·5N-hydrochloric acid, 30·5:10·1:17·7 (Laland, Overend & Webb, 1952); (11) propanol–water, 4:1 (Greenberg, 1952); (12) propanol–water, 3:2 in an ammonia atmosphere (Greenberg, 1952); (13) butanol–formic acid, 95:5.

Of these solvent systems, nos. 1–5 were used for the separation of amino acids, no. 6 for pteridines, no. 7 for pyrimidines, nos. 7–10 for purines and pyrimidines, and nos. 11 and 12 for 5-amino-4-imidazolecarboxamide riboside. System 2 was also used for the separation of ribose and 5(4)-amino-4(5)-imidazolecarboxamide from the hydrolysis products of the riboside. In certain experiments, the keto acids isolated from the ether-soluble metabolic products in the medium at the end of the culture period were separated by direct chromatography in solvent system 13 (Wieland & Fischer, 1949; Magasanik & Umbarger, 1950); in others, separation was achieved by the method of Alfthan & Virtanen (1955), the amino acids obtained on reduction of the 2:4-dinitrophenylhydrazones being identified by chromatography in solvent system 2 in parallel with authentic amino acids as reference compounds. Solvent system 6 was used in conjunction with solvent system 2 for two-dimensional chromatography of protein hydrolysates according to the method of Levy & Chung (1953).

In general, the chromatograms were prepared on Whatman no. 54 paper. The use of other papers (Whatman no. 52 or no. 1) is specifically mentioned in the Results section. Amino acids were located on the developed chromatograms by spraying with 0·5% (w/v) ninhydrin in ethanol containing acetic acid (5%, v/v); purines, pyrimidines, pteridines and 5-amino-4-imidazolecarboxamide riboside by their absorption or fluorescence in ultraviolet light at 254 or 365–366 mμ. Ribose and 5(4)-amino-4(5)-imidazolecarboxamide were detected with aniline hydrogen phthalate (Partridge, 1949) and the Pauly reagents (Block, 1951) respectively.

Accumulation of amino acid in cultures treated with aminopterin. This was demonstrated by direct chromatography of culture filtrates in solvent systems 1 and 2. As relatively large amounts (120 μl.) of the filtrates were necessary for the detection of the amino acids, the culture supernatants in certain experiments were concentrated *in vacuo*, then diluted with methanol (3 vol.), and filtered and again concentrated before the application of equivalent amounts to the paper. As observed by Dagley, Dawes & Morrison (1950), however, precipitation of salts in this way resulted in some loss of ninhydrin-positive material. For the estimation of amino acid concentration the culture supernatants were run in parallel with known amounts of the appropriate reference compounds. After locating the amino acids with ninhydrin, areas containing the spots were cut from the paper and weighed on a microbalance before extraction of the colour with 60% ethanol in order to correct for the variable 'paper blank' (cf. Block, LeStrange & Zweig, 1952).

Chemical fractionation and analysis of Aerobacter aerogenes. The cells from each 100 ml. of medium were transferred to a 15 ml. Pyrex centrifuge tube, and washed three times with water and then extracted three times with 5% (w/v) trichloroacetic acid (5 ml.) at 0°. The cell residue was then washed with ethanol until the washings were free from trichloroacetic acid, and boiled for 1 hr. with 95% (v/v) methanol (5 ml.) to remove extractable fats. This extraction was repeated and material was then dried with ethanol and ether. In initial experiments nucleic acids were removed from the cell residue by extraction three times with 5% (w/v) perchloric acid at 90° (Ogur & Rosen, 1950). As observed by Bolton, Abelson & Aldous (1952), however, relatively large amounts of protein were removed with the nucleic acids under these conditions, and for most of the experiments described in this paper 5% (w/v) trichloroacetic acid (Schneider, 1945) was used in place of perchloric acid.

After the extraction of the nucleic acids the material was again dried with ethanol and ether. The centrifuge tube containing this residue was then drawn out near the open end before the addition of 6N-hydrochloric acid (0·5 ml.). The tube was flushed with nitrogen, and sealed and placed in a metal cylinder in an oven at 110° for 22 hr. to hydrolyse the protein. Hydrochloric acid was removed from the hydrolysate by the repeated addition and evaporation of water under reduced pressure, and then by concentration *in vacuo* at room temperature over phosphorus pentoxide and potassium hydroxide. The residue thus obtained was made to 5 ml. with water and portions were removed for the determination of total nitrogen. The remainder was again evaporated *in vacuo* and reserved for paper or ion-exchange chromatography.

Phosphorus was determined by the method of Berenblum & Chain (1938) and nitrogen by the micro-Kjeldahl method, the apparatus described by Markham (1942) being used for the distillation of the ammonia. Deoxyribonucleic acid and ribonucleic acid contents of the hot perchloric acid or trichloroacetic acid extracts were measured by the Dische (1930) and Euler & Hahn (1946) methods respectively, purified samples of the corresponding nucleic acids of herring roe and yeast being used as standards. In the determination of extractable phospholipid phosphorus portions of the combined ethanol and ethanol–ether extracts were evaporated from water (2 ml.) in micro-digestion flasks before the addition of 60% (w/w) perchloric acid (1 ml.).

Separation of the amino acids from protein hydrolysates. The acidic and neutral amino acids of hydrolysates of *A. aerogenes* protein were separated on Dowex 50 according to the method of Moore & Stein (1951). The column used generally gave a satisfactory separation of all amino acids other than glycine and cystine, which were eluted together.

For analytical purposes a portion of the hydrolysate containing 150–200 μg. of N was applied to the column; this was increased to 600–1000 μg. when the column was used to separate the amino acids. Under the latter conditions,

alternate effluent fractions were analysed by the ninhydrin method (Yemm & Cocking, 1955) to locate the individual components. From the remaining fractions the contents of the appropriate tubes were combined and deionized on small columns (15 cm. × 0·8 cm.²) of Zeo-Karb 215 (H^+) according to the method of Alfthan & Virtanen (1955). The amino acid eluates were evaporated to dryness on a steam bath, the residues dissolved in a little water and portions of these solutions checked for purity by paper chromatography in solvent system 2. For the separation of glycine from the small amount of cystine present in hydrolysates prepared by the foregoing method, the whole fraction containing both of these amino acids was applied as a streak across the width of a 25 mm. strip of Whatman no. 52 paper. After development for 48 hr. in solvent system 2, the glycine, located by cutting a lateral strip 3 mm. wide from the paper and treating this with ninhydrin, was eluted in water.

In experiments in which valine and leucine were the only amino acids to be isolated, practically quantitative recovery was obtained, since their location in the effluent fractions could be established from the analysis of the first 150 fractions from the column and reference to previous runs.

Hydrolysis of the nucleic acid fractions and isolation of the purine and pyrimidine bases. The extracts from the above fractionation, containing both ribo- and deoxyribonucleotides, were heated in boiling water until free from trichloroacetic acid (30–60 min.), then made N with respect to sulphuric acid and hydrolysed for 1 hr. at 100°. The purine bases and pyrimidine nucleotides thus liberated were separated as described by Kerr, Seraidarian & Wargon (1949). Adenine and guanine were isolated as the hydrochlorides from the purine fraction by chromatography on columns of Dowex 50 (H^+) (Cohn, 1949), and recrystallized from 0·1 N-hydrochloric acid. The extracts containing the pyrimidine nucleotides were evaporated *in vacuo* over phosphorus pentoxide and potassium hydroxide, and the residual solids hydrolysed with 72% (w/v) perchloric acid (Marshak & Vogel, 1951) at 100°. The hydrolysates were diluted with water, and neutralized with potassium hydroxide and filtered to remove potassium perchlorate. The filtrates were evaporated under reduced pressure, and centrifuged and taken to dryness *in vacuo* over phosphorus pentoxide. The residues were dissolved in water and the solutions applied as streaks to sheets of Whatman no. 54 paper. The chromatograms were developed for 7 hr. in solvent system 7. Uracil (R_F 0·30), cytosine (R_F 0·05) and thymine (R_F 0·50) were located by examination through a willemite-coated quartz plate (Marsh, 1954) of the dry chromatograms mounted in front of a grid-type low-pressure mercury-vapour lamp (Thermal Syndicate Ltd., Wallsend, Northumberland, ref. T/M5/535) emitting high-intensity radiation at 254 mμ. Strips containing the bases were cut from the chromatograms and eluted with 0·1 N-hydrochloric acid. The eluates were concentrated *in vacuo* over phosphorus pentoxide and potassium hydroxide until crystallization occurred,

Radioactive amino acids. L-[$^{14}C_3$]Alanine, L-[$^{14}C_5$]valine and DL-[1-^{14}C]valine were purchased from The Radiochemical Centre, Amersham, Bucks.

Location and measurement of radioactivity. Radioactive compounds on paper chromatograms were located by autoradiography on Crystallex and Industrex X-ray film (Kodak Ltd.) with an exposure of 21–28 days. Solid samples were assayed for activity on flat aluminium or glass dishes (2 cm. diam.) with an assembly consisting of an end-window, Geiger–Müller tube (General Electric Co. Ltd., Magnet House, Kingsway, London, W.C. 2; type EHM 2ª) and Dynatron scaler (type 200, Dynatron Radio Ltd., Maidenhead, Berks). Carbon dioxide, either metabolic or from chemical degradations, was converted into barium carbonate, samples of which were counted to within 5% standard error and corrected for self-absorption. A correction for self-absorption was also applied when activity measurements were made on derivatives (e.g. 2:4-dinitrophenylhydrazones), whereas other compounds (e.g. amino acids, purines and pyrimidines) were plated at low concentrations (less than 100 μg./cm.²) and were not so corrected.

Chemical degradation of labelled amino acids was done by established methods, references to which are given in the Results section. For the detection and degradation of formic acid, Pirie's (1946) method was used. The carbon dioxide thus obtained was trapped in N-sodium hydroxide and isolated as barium carbonate. To eliminate the possibility of the contamination of the latter with traces of volatile, radioactive fatty acids, the barium carbonate was decomposed with hydrochloric acid at 0° and the evolved carbon dioxide swept with a current of CO_2-free air through a trap cooled in ethanol–solid CO_2 into 0·2 N-barium hydroxide. The barium carbonate residues from these experiments were pooled and used as a source of $^{14}CO_2$ for the growth experiments summarized in Table 9.

Special chemicals. The aminopterin and leucovorin (calcium salt) used in the following experiments were gifts from Dr Nickerson, Rutgers University. The sample of synthetic 5(4)-amino-4(5)-imidazolecarboxamide, used as a reference compound, was kindly provided by Dr D. W. Woolley, of the Rockefeller Institute, New York. Coenzyme A (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) and pantothenic acid were purchased from V. A. Howe and Co., 46 Pembridge Road, London, W. 11, and Roche Products Ltd., Welwyn Garden City, respectively.

RESULTS

Identification of alanine and valine in cultures of Aerobacter aerogenes *treated with aminopterin.* Direct chromatography in solvent system 1 of supernatants from *A. aerogenes* cultures incubated for 12–16 hr. in the presence of aminopterin (1:12 000) revealed two amino acids of R_F values 0·145 and 0·29. These were not observed either in filtrates from control cultures incubated for the same period in the absence of the antagonist, or among the soluble components released when washed suspensions of cells from both experimental and control cultures were disintegrated at room temperature in a Mickle shaker (total shaking time 15 min., in five equal periods with 3 min. rest between each period). To isolate the amino acids from the experimental cultures the medium (100 ml.) was partially desalted with methanol as outlined under Materials and Methods and then chromatographed on four sheets of Whatman no. 54

paper in solvent system 2. The amino acids, located by spraying lateral strips from each chromatogram with ninhydrin, were eluted with water and the eluates evaporated *in vacuo*. These were rechromatographed by the ascending method on Whatman no. 1 paper in solvent system 6 to eliminate pteridines (detected by their fluorescence in ultraviolet light), which were derived from impurities and breakdown products of the aminopterin. The amino acids (R_F 0·38 and 0·60) thus isolated were identified as alanine and valine by co-chromatography with the authentic compounds in solvent systems 1–5. As the R_F values of valine in solvent systems 1, 2 and 5 were similar to those of methionine, differentiation between these amino acids was achieved by treatment with hydrogen peroxide (Dent, 1948).

For chemical characterization, the amino acids, separated from the culture medium (300 ml.) as above, were decomposed with chloramine-T (Ehrensvärd, Reio, Saluste & Stjernholm, 1951). The product from the degradation of the slow-moving component (R_F 0·38 in solvent system 6), distinguished from threonine by its greater resistance to periodic acid, was removed in a current of air and trapped in 2:4-dinitrophenylhydrazine (half-saturated solution in 2N-hydrochloric acid). The crystalline product which separated was recrystallized from aqueous ethanol to yield acetaldehyde 2:4-dinitrophenylhydrazone, m.p. 154° (uncorr.), alone and in admixture with the authentic compound. The volatile product, similarly separated from the decomposition of the second component (R_F 0·60 in solvent system 6) was trapped in 0·05N-potassium permanganate–0·05N-sodium bicarbonate. This solution was acidified and extracted with ether. Evaporation of the ether yielded an oil, from which an anilide was prepared in the usual way. The product, recrystallized first from water and then from aqueous ethanol, formed needles of m.p. 105° (uncorr.) alone and in admixture with the anilide of *iso*butyric acid similarly prepared from authentic DL-valine.

Accumulation of extracellular alanine and valine was also observed to follow the addition of aminopterin to cultures of *Escherichia coli* and *Aerobacter cloacae* in the early logarithmic phase of multiplication. In contrast with *A. aerogenes*, however, control cultures of these bacteria grown in the absence of the antagonist also contained detectable amounts of alanine and valine. The presence of these and other amino acids in filtrates from cultures of *E. coli* in a simple chemically defined medium has been described previously by Dagley & Johnson (1956).

In cultures of *A. aerogenes* traces of valine were present in the culture medium 1 hr. after the addition of aminopterin. At this stage of the culture alanine was not detected. In samples removed after a further 90 min., however, both amino acids were present. As judged by the intensities of the colours formed on the developed chromatograms with ninhydrin, the concentrations of alanine and valine in the medium then increased progressively with time until 21 hr., after which recovery from the inhibition of growth began. Six hours after the onset of recovery, when the turbidity of the experimental culture had increased from 35 to 94 % of that of the fully-grown control, as well as during the subsequent increase in turbidity from 94 to 120 %, neither alanine nor valine was demonstrable by chromatography of portions of the culture medium. In three experiments in which the concentrations of the accumulated amino acids were determined at or near the estimated time of recovery, values of 2·45, 2·0 and 1·75 μmoles/ml. and 2·75, 2·5 and 1·76 μmoles/ml. were obtained for alanine and valine respectively.

Effect of aminopterin on the chemical composition of Aerobacter aerogenes. To determine if the accumulation of alanine and valine in the aminopterin-treated cultures was reflected in an alteration in either content or amino acid composition of the total cellular protein, cells from inhibited and control cultures were analysed as described under Materials and Methods. Results of these analyses are summarized in Tables 1 and 2.

As shown in Table 1, the amounts of the various cell components were measured relative to the deoxyribonucleic acid phosphorus (DNA-P) content, since this method generally provides the most suitable indication of the average cell composition and avoids difficulties inherent in the interpretation of per cent dry wt. values (e.g. Davidson & Leslie, 1950*a*, *b*). It is apparent, however, from the results expressed in this way, that growth inhibition by aminopterin in *A. aerogenes*, as in *E. coli* (Webb & Nickerson, 1956), *Lactobacillus casei* (Prusoff, Teply & King, 1948) and *Lactobacillus leichmanii* (Rege & Sreenivasan, 1954), causes a decrease in DNA-P content which is greater than that in any other cell component. Thus comparison of the ratios of DNA-P/ribonucleic acid phosphorus (RNA-P) in the two preparations from the inhibited cultures (E_1 and E_2) with the corresponding controls (C_1 or C_2 and C_3) reveals reductions in DNA-P content of 50 and 36 % respectively. These figures may represent minimum values, since the RNA-P content has been assumed to remain unaltered, whereas an analogous decrease (51 %) in DNA content of *L. leichmanii* under partial aminopterin bacteriostasis (0·25 μg./ml.) is accompanied by a 23 % reduction in the amount of RNA (Rege & Sreenivasan, 1954).

Relative to RNA-P, a small decrease in protein content is induced in *A. aerogenes* by aminopterin

Table 1. *Effect of aminopterin on the chemical composition of* Aerobacter aerogenes

Analyses are recorded for *A. aerogenes* cells harvested during the growth inhibition which followed the addition of aminopterin (83 μg./ml.) to cultures in the early logarithmic phase (turbidity 0·20≡85 μg. dry cell wt./ml.) (preparations E_1 and E_2), and after recovery from this inhibition (preparation E_R), together with the corresponding control preparations (C_1, C_2, C_3 and C_4) from cultures grown in the absence of the antagonist.

	E_1	C_1	C_2	E_2	C_3	E_R	C_4
Age of culture (hr.)	19	19	19	20	20	44	44
Time (hr.) of aminopterin addition	3	—	—	2·5	—	2·5	—
Cell density at time of harvesting (μg. dry cells/ml.)	200	665	660	260	658	612	628
Acid-soluble N/DNA-P	—	—	—	3·9	3·3	5·5	6·0
Acid-soluble P/DNA-P	12·1	6·23	6·48	6·5	5·5	4·0	3·7
RNA-P/DNA-P	9·6	4·7	5·1	7·1	4·5	4·0	3·1
Protein N/DNA-P	30·7	—	20·5	24·6	19·6	21·0	20·2
Acid-soluble N/RNA-P	—	—	—	0·6	0·7	1·4	1·9
Acid-soluble P/RNA-P	1·3	1·3	1·3	0·9	1·2	1·0	1·2
DNA-P/RNA-P	0·10	0·21	0·19	0·14	0·22	0·25	0·33
Protein N/RNA-P	3·2	—	4·0	3·4	4·0	5·5	6·6

Table 2. *Amino acid composition of* Aerobacter aerogenes *protein*

Contents of acidic and neutral amino acids in hydrolysates of the protein from aminopterin-inhibited *A. aerogenes* (preparation E_2) and the corresponding control cells (preparation C_3), determined according to the method of Moore & Stein (1951). Separation of alanine from glycine and cystine was incomplete.

Amino acid	Amino acid residues (μmoles/100 μg. of protein N) E_2		Amino acid residues (μmoles/100 μg. of protein N) C_3	
Aspartic acid	0·60		0·62	
Threonine	0·32		0·32	
Serine	0·28		0·28	
Glutamic acid	0·62		0·68	
Alanine	0·45	1·19	0·56	1·14
Glycine + cystine	0·74		0·58	
Valine	0·42		0·44	
Methionine	0·08		0·09	
Isoleucine	0·30		0·29	
Leucine	0·48		0·52	

Table 3. *Incorporation of* ^{14}C *from generally-labelled* L-*alanine and* L-*valine by* Aerobacter aerogenes *during recovery from inhibition by aminopterin*

Total activity (counts/min.)

	Cultures supplemented before recovery with $[^{14}C_3]$Alanine	Cultures supplemented before recovery with $[^{14}C_5]$Valine
Initial activity added to culture medium	$1·92 \times 10^5$	$1·73 \times 10^5$
Residual activity in medium after recovery	420	3400
Cell fractions:		
Acid-soluble components	1620	690
Lipids	1950	2160
Nucleic acids	$1·92 \times 10^4$	$0·81 \times 10^4$
Protein	$2·70 \times 10^4$	$6·00 \times 10^4$

(Table 1). This is not associated, however, with any significant change in the amino acid composition of the total cell protein (Table 2).

With recovery from inhibition by aminopterin, the chemical composition of *A. aerogenes* becomes similar to that of normal cells grown in the absence of the antagonist (Table 1). As is to be expected from the limited viability of fully grown normal cultures of *A. aerogenes* in the simple chemically defined medium, cells harvested 44 hr. after inoculation (i.e. some 30–36 hr. after the end of logarithmic multiplication) differ significantly in chemical composition from cells isolated after 20 hr. (Table 1). The fact that the chemical composition of cells after recovery from the inhibitory effects of aminopterin appears to be intermediate between those of the 20 and 44 hr. control preparations, probably reflects nothing more than the absence of the appropriate control to the recovery culture. It is significant, however, that cells after recovery have essentially normal ratios of RNA-P/DNA-P and protein N/DNA-P.

Utilization of L-*alanine and* L-*valine by* Aerobacter aerogenes *on recovery from inhibition by aminopterin.* To investigate the metabolism of the accumulated alanine and valine during the recovery phase, aminopterin-treated cultures of *A. aerogenes* at the end of the period of growth inhibition were supplemented separately with each of these amino acids generally labelled with ^{14}C. After recovery, the cells were harvested and fractionated. The total ^{14}C-activities in the crude fractions thus separated are given in Table 3. The figures shown should be regarded as approximate rather than absolute values, as, for example, no allowance has been made for the contamination of the nucleic acid fractions with protein, whereas the activities of the protein components have been calculated from measurements made on samples of

the insoluble residues obtained on completion of the fractionation.

Under the conditions of these experiments considerable radioactivity from both [$^{14}C_3$]alanine and [$^{14}C_5$]valine was incorporated into the cell protein. The association of this uptake with recovery from the growth inhibition was shown by the absence of ^{14}C-activity from cells harvested during the inhibition phase of an aminopterin-treated culture to which DL-[1-^{14}C]valine (5 μmoles, 0·01 mc) had been added 2 hr. after the addition of the antagonist.

Table 4. *Specific activities of certain amino acids isolated from the proteins of* Aerobacter aerogenes *after recovery from aminopterin inhibition in the presence of* L-*alanine or* L-*valine generally labelled with* ^{14}C

Amino acid	Specific activity (counts/min./μmole) after recovery in the presence of [$^{14}C_3$]Alanine	[$^{14}C_5$]Valine
Valine	0	1340
Alanine	1312	—
Leucine	186	1070
Aspartic acid	292	—
Threonine	306	—
Serine	314	—
Glutamic acid	470	—
Phenylalanine	178	—
Isoleucine	—	360

When recovery occurred in the presence of generally labelled L-alanine and L-valine, neither amino acid was detected in the medium at the end of the culture period. In each experiment the corresponding keto acids, pyruvic acid and 3-methyl-2-oxobutanoic acid (α-oxo*iso*valeric acid), were present, however, and accounted for much of the residual activity. The incorporation of ^{14}C-activity into the lipids and, at this stage in the investigation, into the nucleic acids, was not investigated further. The distribution of activity among the amino acid components of the cell protein was studied initially by autoradiography of two-dimensional chromatograms of acid hydrolysates of this fraction. The results obtained were as follows: when recovery occurred in the presence of generally labelled L-alanine, ^{14}C-activity was present to a high level in the alanine of the cell protein and to a lesser but still marked extent in glutamic acid, glycine, serine, threonine, aspartic acid, leucine, lysine, phenylalanine, cystine and histidine. When recovery occurred in the presence of generally labelled L-valine, however, significant activity was incorporated only into the valine and leucine components of the protein. This finding is in agreement with the work of Abelson & Vogel (1955), in which the addition of [^{14}C]valine to cultures of *Neurospora crassa* and *Torulopsis utilis* resulted in significant labelling of protein-bound

Table 5. *Distribution of* ^{14}C *in certain amino acids isolated from the proteins of* Aerobacter aerogenes *after recovery from aminopterin inhibition in the presence of either* L-*alanine or* L-*valine generally labelled with* ^{14}C

Amino acid	Carbon atom or radical	Method of degradation	Activity (% of total activity) after recovery in the presence of [$^{14}C_3$]Alanine	[$^{14}C_5$]Valine
Serine	$C_{(1)}$	Sakami (1950)	50·1	—
	$C_{(2)}$		45·6	—
	$C_{(3)}$		2·2	—
Threonine	$C_{(1)}$	Ehrensvärd, Reio, Saluste & Stjernholm (1951)	30·3	—
	$C_{(2)}$		36·3	—
	$C_{(3)}+C_{(4)}$		29·3	—
Aspartic acid	$C_{(1)}$	Oxidation with chloramine-T (Ehrensvärd *et al.* 1951)	29·3	—
	$C_{(1)}+C_{(4)}$	Ninhydrin decarboxylation (Van Slyke, MacFadyan & Hamilton, 1941)	42·6	—
Phenylalanine	$C_6H_5 \cdot CH_2$-	Oxidation to benzoic acid with alkaline permanganate	0	—
Leucine	$C_{(1)}$	Ninhydrin decarboxylation (Van Slyke *et al.* 1941)	46·0	0
	$C_{(2)}$	Deamination with nitrous acid and oxidation with permanganate to *iso*valeric acid, which was then degraded by the Schmidt reaction (Phares, 1951)	50·6	0·26
	$CHMe_2 \cdot CH_2$-	Total activity $-(C_{(1)}+C_{(2)})$	3·4	99·7
Valine	$C_{(1)}$	Ninhydrin decarboxylation (Van Slyke *et al.* 1941)	—	22·7

valine and leucine only. Of the remaining amino acids in the hydrolysate of the *A. aerogenes* protein, only isoleucine, separated from leucine by chromatography for 96 hr. in solvent system 4, possessed activity sufficient to be detected by autoradiography.

The specific activities of certain acidic and neutral amino acids, separated from each protein hydrolysate by ion-exchange chromatography (Moore & Stein, 1951), are given in Table 4. After these determinations, a number of the isolated amino acids were supplemented with the corresponding unlabelled compounds and degraded by established methods, adapted where necessary to the micro scale, to locate the active carbon atoms. The results of these experiments are summarized in Table 5.

Effect of coenzymes and vitamins on inhibition by aminopterin. The results presented in Table 4 suggested the possibility that the accumulation of valine in the aminopterin-treated cultures was due to an inhibition of leucine synthesis, a process which appears to be either directly or indirectly dependent upon folic acid (Woods, 1953). Degradation of the isolated leucine, however, established that the carbon skeleton of this amino acid was not derived from that of valine by the addition of a C_1 unit, as might be expected if a folinic acid-dependent, aminopterin-sensitive reaction was involved in the synthesis. Thus the α-carbon of leucine contained insignificant ^{14}C-activity, whereas the carboxyl carbon of the isolated valine contained 22·7% of the total activity of the molecule (Table 5). In addition, attempts to prevent the growth-inhibitory action of aminopterin on *A. aerogenes* by the addition to the culture medium of L-leucine in concentrations up to 5 μmoles/ml. were unsuccessful. As it thus seemed unlikely that aminopterin had a direct action on the formation of leucine, a number of experiments were done to investigate the possibility of an indirect effect of the antagonist on this synthesis through the inhibition of the production of an essential coenzyme.

It is known that 3-methyl-2-oxobutanoic acid, the precursor of valine, is also a precursor of the pantoic acid moiety of coenzyme A (CoA) in *E. coli*, *A. aerogenes* (Maas & Vogel, 1953) and *Brucella abortus* (Altenbern & Ginoza, 1954). Furthermore, the hydroxymethylation of 3-methyl-2-oxobutanoic acid to α-oxopantoic acid requires *p*-aminobenzoic acid (Purko, Nelson & Wood, 1953, 1954) or folic acid (e.g. Johnson, 1955). Recent work by T. Wainwright & D. D. Woods (personal communication) suggests that the biosynthesis of leucine may occur during the conversion of *iso*-butyryl-CoA and acetyl-CoA. Thus the inhibition of CoA synthesis theoretically could result both directly and indirectly in the accumulation of 3-methyl-2-oxobutanoic acid from which valine would be derived by transamination. Indeed, an accumulation of valine through an inhibition of CoA synthesis has been observed by Maas & Vogel (1953) in a pantoic acid-requiring mutant of *E. coli* when grown in the presence of limiting amounts of pantoic acid. In addition, if the acetyl-CoA required for leucine synthesis according to the above mechanism is derived from pyruvate (e.g. Chantrenne & Lipmann, 1950) a deficiency of the coenzyme could also result in the accumulation of alanine. The addition of pantothenic acid (3 μg./ml.) or coenzyme A (0·3 μg./ml.) to cultures of *A. aerogenes* either before or together with aminopterin, however, was without effect upon either the growth-inhibition or the accumulation of alanine and valine. Although *A. aerogenes* may be impermeable to coenzyme A, it seems unlikely that the lack of effect of pantothenic acid could be due to its inability to enter the cells, since this compound has been shown to prevent the inhibition of growth produced by sulphanilamide in cultures of *Bacillus linens* containing limiting amounts of *p*-aminobenzoic acid (Purko *et al.* 1953).

In further experiments, thiamine, riboflavin, nicotinic acid, biotin, cocarboxylase and pyridoxin at several concentrations (2, 5 and 10 μg./ml.) were found also to be without effect upon either the inhibition of growth or accumulation of amino acid in the aminopterin-treated *A. aerogenes* cultures. The reasons underlying the choice of these substances are to be found in the work of Lascelles & Woods (1954), Lascelles, Cross & Woods (1954), Blakley (1955), Kitahara & Fukui (1955), Montfoort (1955), Ekladius & King (1956), Guthrie, Hillman & Hyatt (1956), Trevelyan & Harrison (1956) and Wright & Stadtman (1956). Partial reversal of the inhibition was obtained, however, with leucovorin, even if this was added several hours after the antagonist, although high concentrations were required to produce a consistent effect. Thus when leucovorin and aminopterin in the ratio of 50:1 were added to a culture of *A. aerogenes* in the early logarithmic phase, the inhibition of growth after a further 6 hr. still amounted to about 60% of that produced by aminopterin (50 μg./ml.) alone. This partial reversal, however, was accompanied by a corresponding decrease (35–40%) in the amounts of alanine and valine accumulated in the medium.

Prevention of inhibition by aminopterin. The inhibitory action of aminopterin (1:24 000) was almost entirely prevented (Fig. 1) by the addition to the simple growth medium of the following: L-serine, L-isoleucine, L-leucine, L-valine, glycine, L-histidine, L-tryptophan, L-glutamic acid, L-threonine, L-methionine, L-lysine, adenine and thymine at mM-concentrations. Furthermore, in

the presence of these supplements, the formation of filamentous cells normally observed in the aminopterin-treated cultures (Nickerson & Webb, 1956) was entirely prevented. The choice of these substances was determined in part by the knowledge that folinic acid is required for the synthesis of many of them, and in part from the distribution of ^{14}C-activity observed in the above experiments with the labelled alanine and valine. In addition, L-isoleucine and L-valine were included to obviate the possible growth-inhibitory effect of L-leucine alone (e.g. Gladstone, 1939; Rowley, 1953).

As a result of many growth experiments with media lacking one or more of the above components, it was possible to eliminate the following as being of secondary importance in the prevention of the inhibition: L-serine, L-isoleucine, L-leucine, L-valine, L-histidine, L-tryptophan, L-methionine, L-lysine and thymine. Thus in the presence of aminopterin the omission of these compounds singly from the medium produced little change in the form of the growth curve from that observed with the complete supplement, although when all were omitted together, their cumulative effects upon the inhibition were apparent (Fig. 2, cf. Fig. 1). The effects of the remaining four (adenine, L-threonine, L-glutamic acid and glycine) were studied in a factorial experiment, the design of which together with the analysis of the data is given in the Appendix. The results obtained revealed adenine to be the active compound in the prevention of the inhibition. This finding has a parallel in the observation of Guthrie *et al.* (1956) that adenine blocks the inhibitory action of amethopterin (4-amino-4-deoxy-10-methylpteroylglutamic acid) on *Bacillus subtilis* in a minimal medium. The presence of the purine also reduced by 50–70 % the amounts of alanine and valine chromatographically detectable in portions (120 μl.) of medium from aminopterin-treated cultures, although complete suppression of amino acid accumulation under the conditions of these experiments was not observed.

Hypoxanthine was as effective as adenine in preventing the inhibition due to aminopterin, whereas guanine at a concentration limited by solubility to 7 μg./ml. was not only inactive but also appeared to inhibit the action of adenine. This observation contrasts with the known ability of normal *A. aerogenes* cells to utilize guanine, although less effectively than adenine (Hamilton, Brown & Stock, 1952; Balis, Brooke, Brown & Magasanik, 1956). It is possible, however, that

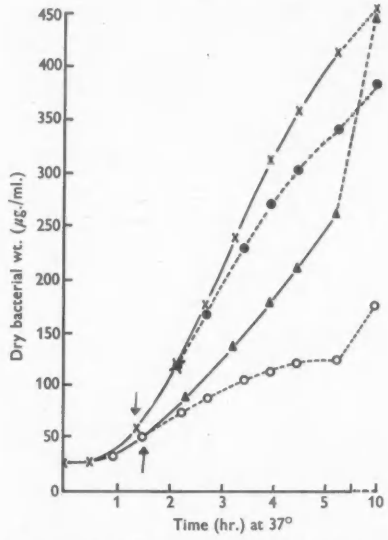

Fig. 1. Curves show the growth of cultures of *A. aerogenes* in the simple chemically defined medium with (×) and without (▲) a supplement of L-serine, L-isoleucine, L-leucine, L-valine, glycine, L-histidine, L-tryptophan, L-glutamic acid, L-threonine, L-methionine, L-lysine, adenine and thymine (all mM). ●, ○, Effect of the addition of aminopterin (final concentration 1:24 000; about 0·1 mM) to cultures with and without supplement respectively at the times indicated by the arrows.

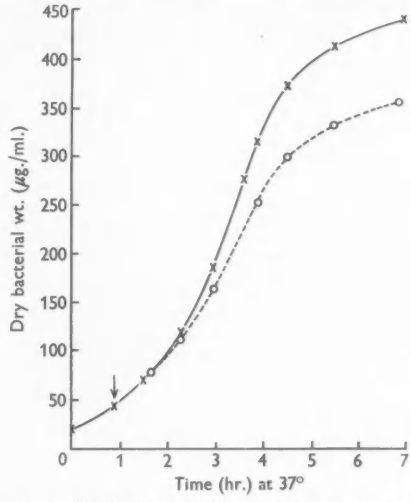

Fig. 2. ○, Effect of the addition (at time indicated by arrow) of aminopterin to a concentration of 0·1 mM to a culture of *A. aerogenes* in Monod's medium supplemented with adenine, L-threonine, L-glutamic acid and glycine (all mM). ×, Growth of the control culture in the same medium in the absence of the antagonist.

under conditions of purine deficiency, guanine may inhibit the utilization of adenine, as observed by Aaronson (1955) in cultures of the purine-requiring *Staphylococcus flavocyaneus*.

Accumulation of 5-amino-4-imidazolecarboxamide riboside in aminopterin-treated cultures of Aerobacter aerogenes. The prevention by adenine both of the growth inhibition and the production of alanine and valine in the treated cultures, when considered together with the decreased deoxyribonucleic acid content of the cells (Table 1), suggested the possibility of a relationship between amino acid accumulation and inhibition of purine synthesis. In cultures of certain bacteria inhibition of purine synthesis by the folic acid antagonists, as well as by sulphonamides, is known to result in the accumulation of 5-amino-4-imidazolecarboxamide riboside (Woolley & Pringle, 1950; Edwards, Skipper & Johnson, 1952; Stetten & Fox 1945; Ravel *et al.* 1948; Greenberg, 1952). In previous work (Webb, 1955) attempts to demonstrate the presence of this imidazole derivative in cultures of *A. aerogenes* after recovery from inhibition by aminopterin were unsuccessful. In the present studies, however, when cultures in the inhibition phase were examined, high levels of 5-amino-4-imidazolecarboxamide riboside were detected. This compound was isolated with a yield of 1 mg./100 ml. of medium, by Greenberg's (1952) method, as this, in contrast with the most recent procedure (Greenberg & Spilman, 1956), gave an efficient and convenient elimination of pteridines derived from the aminopterin preparation. The product thus obtained contained equimolecular proportions of pentose and non-acetylatable diazotizable amine, the latter being determined by the method of Bratton & Marshall (1939) after treatment with acetic anhydride (Rosenthal & Bauer, 1939). The compound was characterized by its absorption spectra in 0·1 N-hydrochloric acid ($\lambda_{max.} = 246$ and 268 mμ; $\epsilon_{268\ m\mu} = 11\,560$) and 0·1 N-sodium hydroxide ($\lambda_{max.} = 267$ mμ; $\epsilon_{267\ m\mu} = 14\,250$), in agreement with the data of Greenberg & Spilman (1956), and by its hydrolysis (0·5 N-hydrochloric acid at 100° for 1 hr.) to 5(4)amino-4(5)-imidazolecarboxamide and ribose.

Incorporation of the carboxyl carbon of [1-^{14}C]*valine into the nucleic acids of* Aerobacter aerogenes. As glycine and aspartic acid, both of which can be derived from alanine (Table 4), are known precursors of purines and pyrimidines in bacteria as in mammalian tissues (see review by Carter, 1956), the utilization of labelled alanine by *A. aerogenes* would be expected to result in the incorporation of radioactivity into the nucleic acids by mechanisms other than those dependent upon folinic acid. For this reason subsequent experiments to investigate the relation between amino acid accumulation and purine synthesis were done with DL-[1-^{14}C]valine. The labelled amino acid was added (*a*) in tracer amounts to a normal culture of *A. aerogenes* in the early logarithmic phase of multiplication, and (*b*) towards the end of the period of growth-inhibition in an aminopterin-treated culture as a supplement to the accumulated valine. This approach was used to determine if differences in utilization would result from the high level of exogenous valine in the inhibited culture (cf. Koch, 1955). A similar distribution of radioactivity was observed, however, in the fractions separated from cells grown under the conditions of (*a*) or (*b*). In consequence of this and of the limitation imposed on the scale of experiment (*b*) by the amount of aminopterin available, only the results obtained with *A. aerogenes* grown in the presence of tracer amounts of DL-[1-^{14}C]valine are summarized in Table 6.

Table 6. *Distribution of* ^{14}C*-activity in the fractions isolated from* Aerobacter aerogenes *after growth in Monod's medium containing* DL-[1-^{14}C]*valine*

The culture was grown in 1 l. of medium contained in a 5 l. bolt-head flask fitted with an inoculation port, an outlet tube and two inlet tubes, one of which terminated 1 in. above the liquid surface; the second tube reached to the bottom of the vessel. The two inlet tubes were joined externally through a two-way tap with a source of sterile, CO_2-free air, which could thus either be passed through the medium, or used to replace the gas phase in the flask. An arrangement of two-way taps in the outlet tube enabled a trap containing N-sodium hydroxide to be inserted in the train. The medium was brought to 37° and inoculated with 10 ml. of a 16 hr. culture of *A. aerogenes* in the same medium. After 2 hr., CO_2-free air was drawn rapidly through the medium for 15 min., and a solution of DL-[1-^{14}C]valine (0·04 mc) in Monod's medium (5 ml.) added. The sodium hydroxide trap was inserted, and a slow current of air drawn continuously through the gas phase of the vessel for a further 15 hr. The air stream was then directed through the medium for 20 min. to remove dissolved CO_2, after which the culture was centrifuged and the cells were submitted to chemical fractionation. The carbonate formed in the sodium hydroxide trap was precipitated with barium, and washed and assayed for activity.

	Total ^{14}C-activity (counts/min.)
Initial activity in medium	$4{\cdot}6 \times 10^6$
Final activity in medium	
Ether-soluble	$1{\cdot}08 \times 10^5$
Ether-insoluble	$21{\cdot}2 \times 10^5$
Metabolic CO_2	$2{\cdot}64 \times 10^{5*}$
Lipids	$0{\cdot}03 \times 10^5$
Protein	$4{\cdot}7 \times 10^5$
Nucleic acids	$3{\cdot}6 \times 10^5$

* Specific activity = $10{\cdot}2 \times 10^3$ counts/min./m-mole.

Table 7. *Specific activities of certain of the amino acids isolated from a hydrolysate of the cell proteins of* Aerobacter aerogenes *cultivated in the presence of* DL-[1-^{14}C]*valine*

Amino acid	Specific activity (counts/min./μmole)
Aspartic acid	49·2
Serine	50·2
Glutamic acid	46·6
Alanine	12·2
Isoleucine	20·2
Leucine	22·2
Valine	970

Table 8. *Specific activities of the purine and pyrimidine bases isolated from the nucleic acids of* Aerobacter aerogenes *after growth in the presence of* DL-[1-^{14}C]*valine*

Base	Specific activity (counts/min./μmole)
Adenine	420
Guanine	410
Uracil	0
Cytosine	0
Thymine	190

In confirmation of earlier conclusions, autoradiographs of paper chromatograms of hydrolysates of the cell proteins showed no activity in amino acids other than valine. Slight activity was detected, however, in certain of the amino acids separated from a protein hydrolysate by chromatography on Dowex 50 (Moore & Stein, 1951). These results (Table 7) indicate some re-cycling of the metabolic carbon dioxide during the course of the experiment.

Table 6 shows that almost 50% of the total activity initially present remained in the medium at the end of the growth period. As this probably reflects the inability of *A. aerogenes* to utilize the D-isomer of valine, the isolation of this activity was not attempted. The ether-soluble fatty acid components of the culture filtrate were separated by distillation in steam into volatile and non-volatile fractions, which required 0·3 and 1·05 m-mole of sodium hydroxide respectively for neutralization. The volatile fraction contained 0·05 m-mole of formic acid, which had a specific activity of $2{\cdot}7 \times 10^3$ counts/min./m-mole.

From the nucleic acid fraction (mixed ribo- and deoxyribo-nucleotides) adenine and guanine were isolated as the crystalline hydrochlorides by the methods described earlier. Both bases possessed high radioactivity (Table 8), unaltered on chromatography in solvent systems 8–10. In contrast, the pyrimidine bases uracil and cytosine, separated from perchloric acid hydrolysates of the nucleotide fraction by paper chromatography in solvent system 7, were devoid of radioactivity. Thymine, however, had a specific activity approximately half that of adenine or guanine (Table 8), which was unchanged on chromatography in solvent systems 8–10. The location of this activity solely in the methyl group of thymine follows from the known derivation of the latter from uracil, either as the free base as observed in *B. subtilis* (Rege & Sreenivasan, 1954), or more probably as the riboside (Hammarsten, Reichard & Saluste, 1950; Rose & Schweigert, 1953) or deoxyriboside (Friedkin & Roberts, 1956).

Degradation of the isolated guanine was carried out by the method of Brown, Roll, Plentl & Cavalieri (1948) after the addition of inactive guanine sulphate (15·2 mg.) as carrier. The yield of crude guanidine, isolated as the picrate (3·45 mg.), was insufficient for complete purification; nevertheless, after one recrystallization from water the specific activity of the product was 25% of that of the initial guanine. Thus at least 25% of the total activity of the guanine molecule was located in the $C_{(2)}$ atom.

Incorporation of carbon dioxide into the proteins and nucleic acids of Aerobacter aerogenes. In the foregoing experiment a significant percentage of the activity initially supplied to the culture was eliminated as carbon dioxide (Table 6) and it was necessary therefore to determine whether this formed an intermediary for the incorporation of the valine carboxyl carbon into the purine bases and thymine. Although the activities of the purine bases (approx. $4{\cdot}1 \times 10^5$ counts/min./m-mole, Table 8) were greater than that of the metabolic carbon dioxide ($10{\cdot}2 \times 10^3$ counts/min./m-mole, Table 6) evolution of carbon dioxide of considerably higher activity may have occurred during the early stages of logarithmic growth when the rates of synthetic processes were maximal. This would be shown by a maximum in the curve expressing the variation in specific activity of the metabolic carbon dioxide with time, as observed by Andersson-Kottö, Ehrensvärd, Högström, Reio & Saluste (1954) in cultures of *Neurospora crassa* containing $^{13}CH_3{}^{14}CO_2H$. Later, when the culture entered or approached the stationary phase, the formation of unlabelled carbon dioxide from other sources would result in the dilution of the activity of the total carbon dioxide without a corresponding decrease in the activity previously incorporated into cell components.

To investigate the utilization of carbon dioxide by *A. aerogenes* for the synthesis of protein and more particularly of nucleic acids, a culture was grown for 15 hr. in a medium containing $NaH^{14}CO_3$ and in an atmosphere enriched with $^{14}CO_2$. Details of this experiment are summarized in Table 9.

Most of the amino acids separated from a hydrolysate of the cell protein exhibited slight radioactivity, although the total counts recorded for the

Table 9. *Specific activities of certain amino acids and of the nucleic acid bases isolated from* Aerobacter aerogenes *after growth in the presence of* [^{14}C]*carbon dioxide*

A. aerogenes was inoculated into 900 ml. of Monod's medium at 37° contained in the 5 l. bolt-head flask used in the previous experiment (Table 6). After 2 hr. the air inlet to the culture flask was closed, and the flask evacuated by suction from a water pump attached to the outlet tube. Sterile, CO_2-free air was then admitted and the flask again evacuated. A solution of $Na_2{}^{14}CO_3$ [prepared by the absorption of $^{14}CO_2$ from $Ba^{14}CO_3$ (2 g., $6{\cdot}6 \times 10^4$ counts/min./g.) in NaOH, followed by precipitation of the carbonate with ethanol] in Monod's medium (25 ml.) was titrated with M-KH_2PO_4 to pH 7·5 and immediately drawn into the evacuated culture vessel through the longer of the two inlet tubes. $^{14}CO_2$ (generated from 3 g. of $Ba^{14}CO_3$ and collected in a gas burette over N-H_2SO_4) was introduced through the second inlet, and the internal pressure restored to atmospheric by the admission of CO_2-free air. The inlet and outlet leads were closed and the culture was incubated for a further 15 hr. At the end of this time a trap containing N-sodium hydroxide was inserted between the outlet tube and the water pump, and suction applied and the outlet tap opened. After a few seconds the inlet taps were opened, 5 ml. of 5N-H_2SO_4 was drawn into the vessel and CO_2-free air drawn alternately through the medium and the gas phase for 20 min. A total of $4{\cdot}2 \times 10^4$ counts/min. was recovered as barium carbonate from the contents of the sodium hydroxide trap. The ether-soluble components of the culture medium at the end of growth were separated by distillation in steam into the volatile and non-volatile components; these had total activities of 322 and 913 counts/min. respectively. The volatile fraction contained 0·26 m-mole of formic acid of specific activity 920 counts/min./m-mole.

Amino acid	Specific activity (counts/min./m-mole)	Purine/pyrimidine base	Specific activity (counts/min./m-mole)
Aspartic acid	4780	Adenine	9380
Threonine	4600	Guanine	9060
Glutamic acid	3000	Uracil	9510
Isoleucine	4600	Cytosine	8750
Leucine	2440	Thymine	7530

samples (approx. 50 μg.) of valine, alanine and glycine + cystine were only 12–15% greater than background. The incorporation of ^{14}C into the amino acids listed in Table 9 is in agreement with previous work by Ehrensvärd (1948) and Abelson, Bolton & Aldous (1952) with *Torulopsis utilis* and *E. coli*, and the approximately equal activities of aspartic acid, threonine and isoleucine, and the greater activity of aspartic acid over glutamic acid (Table 9) are similar to the findings of Abelson & Vogel (1955) for both *T. utilis* and *N. crassa*.

As observed by Bolton *et al.* (1952) with cultures of *E. coli*, the purine and pyrimidine bases isolated from the nucleic acids of *A. aerogenes* (Table 9) possessed similar specific activities. Although this finding is possibly not immediately reconcilable with the known biosynthetic mechanisms, the significant fact which emerges from the data of Table 9 is that carbon from $^{14}CO_2$ is incorporated into all pyrimidine bases. Thus the fixation of carbon dioxide into formic acid in the anaerobic cultures of *A. aerogenes* (Table 9) is accompanied by a concomitant fixation into the pyrimidines. As the uracil and cytosine of *A. aerogenes* grown in the presence of [1-^{14}C]valine are unlabelled (Table 8), it follows that fixation of metabolic carbon dioxide does not occur to any significant extent under the conditions of the latter experiments.

DISCUSSION

Several examples are available of the accumulation of valine, or its immediate precursor 3-methyl-2-oxobutanoic acid, as a result of specific deficiencies of unrelated amino acids, such as phenylalanine, lysine, proline, histidine and arginine and of vitamins (e.g. biotin) in various microbial mutants which have requirements for these substances (e.g. Mitchell, 1953; Vogel & Bonner, 1954; Davis, 1955; Katsuki, 1955). In addition, an excretion of alanine and an accumulation of pyruvic acid have been observed in wild strains of *E. coli* and *A. aerogenes*, cultivated in simple chemically defined media under various conditions (Dagley *et al.* 1950). Thus the accumulation of alanine and valine in aminopterin-treated cultures of *A. aerogenes* cannot be assumed as evidence that folinic acid-dependent reactions are involved in the metabolism of these amino acids.

It is evident from the results in Table 4 that much of the alanine which accumulates in aminopterin-treated cultures of *A. aerogenes* during the inhibition phase must undergo deamination at the onset of recovery. Thus in cultures supplemented with alanine generally labelled with ^{14}C, the pyruvate formed from the exogenous amino acid is pooled with pyruvate from other sources and provides an intermediate for the synthesis of a number of amino acids. Utilization of pyruvate via the Krebs cycle, which is known to form an important mechanism for synthesis of amino acid in other micro-organisms (e.g. Roberts & Abelson, 1953; Wang, Christensen & Cheldelin, 1953; Abelson & Vogel, 1955; Vavra & Johnson, 1956) would explain the incorporation of activity into aspartic and glutamic acid and the amino acids derived from them.

The distribution of ^{14}C-activity in serine (Table 5) encountered in these experiments, and in particular the low activity of the β-carbon as compared with the α- and carboxyl-carbons, either indicates an extremely high turnover of the hydroxymethyl group, or suggests that this amino acid is not formed directly from pyruvate, but from a C_2 fragment which may be derived from it. Such a mechanism has been postulated in yeast, where serine appears to be synthesized from glycine (Wang *et al.* 1953), although in *E. coli* (Meinhart & Simmonds, 1955), as in mammalian cells (Arnstein & Keglević, 1956), serine forms a precursor of glycine.

Although pyruvate has been shown to be the sole source of valine in *A. aerogenes* (Rafelson, 1955), as in yeast (Strassman, Thomas & Weinhouse, 1953, 1955), no activity is incorporated into this amino acid when recovery from aminopterin inhibition occurs in the presence of generally labelled alanine (Table 4). Thus the valine which accumulates together with alanine in the medium during the inhibition phase must reach a concentration sufficient to prevent its further synthesis during the recovery period. This finding, which provides a further example of the inhibition by an exogenous amino acid of its synthesis (e.g. Ehrensvärd, 1955), has a close parallel in the observations of Adelberg & Umbarger, 1953) that the presence of valine in cultures of *E. coli* inhibits the synthesis not only of the amino acid but also of its precursors.

The synthesis of the carbon chain of valine is of interest for arguments developed later, as it must include the elimination of the carboxyl group from one of the two molecules of pyruvic acid. According to the hypothesis put forward by McManus (1954) and Strassman *et al.* (1953) as a result of experiments with *Torula* yeast, and the work of Umbarger, Brown & Eyring (1957) with a valine-requiring mutant of *E. coli*, acetolactate is an early intermediate in this synthesis. This compound, which is thought to undergo a pinacol-type rearrangement to 3-methyl-2-oxobutanoic acid, may be formed by a ketol condensation between pyruvate and acetaldehyde, the latter being derived by the action of carboxylase on a second molecule of pyruvate (but cf. Wang *et al.* 1953; Vavra & Johnson, 1956).

During the recovery of *A. aerogenes* from inhibition by aminopterin the accumulated valine is used extensively for the synthesis of leucine (Table 4), and provides the *iso*butyl moiety of the carbon skeleton of this amino acid. The remaining atoms, the α- and carboxyl-carbon atoms, are derived from the accumulated alanine via pyruvic acid (Table 5). Leucine biosynthesis in *A. aerogenes* thus appears to be identical with that in *E. coli* (Abelson, 1954), *T. utilis* (Strassman, Locke, Thomas & Weinhouse, 1955; Abelson & Vogel, 1955) and *N. crassa* (Andersson-Kottö *et al.* 1954; Abelson & Vogel, 1955). In these organisms it is known that the leucine carbon chain is formed from the acetyl and *iso*butyryl radicals, although the mechanism of the reaction has yet to be established experimentally. Abelson & Vogel (1955) assume a direct combination, whereas Strassman *et al.* (1955) propose a more elaborate sequence of reactions which includes the condensation of the methyl carbon of acetyl-CoA with α-oxo*iso*valeric acid to yield α-hydroxy-α-*iso*propylsuccinic acid as an intermediary. More recently, T. Wainwright & D. D. Woods (personal communication) have suggested that a condensation occurs between *iso*butyryl-CoA and acetyl-CoA in a manner analogous to the formation of straight-chain fatty acids.

Irrespective of the nature of this condensation reaction, however, it is apparent that at some stage in the biosynthesis of leucine the valine carboxyl carbon is eliminated. The studies with [1-^{14}C]valine (Tables 6–8), considered in relation to those with $^{14}CO_2$ (Table 9), strongly suggest that this carboxyl group is eliminated, not as carbon dioxide, but as some other C_1 unit which is utilized in the synthesis of nucleic acid purines and thymine. Evidence from other systems shows that the source of the thymine methyl carbon atom is either formate, or the β-carbon of serine (Totter, 1954; Elwyn & Sprinson, 1954). It is apparent from Tables 7 and 8, however, that under the conditions of the present experiments the activity incorporated into the entire serine molecule is less than that incorporated into thymine. On this evidence therefore formate appears to be the more likely intermediate in the transfer of the valine carboxyl carbon to thymine.

The fact that the metabolic formate present at the end of logarithmic growth of cultures supplemented with [1-^{14}C]valine contains ^{14}C also suggests that the valine carboxyl carbon forms one source of formate in *A. aerogenes*. The presence of this radioactive formic acid cannot be attributed to the fixation of metabolic carbon dioxide, since it is obvious from the results of Table 9 that, under conditions where formic acid is derived from $^{14}CO_2$, activity is also incorporated into uracil and cytosine. Indeed, the absence of ^{14}C from the pyrimidine bases when [1-^{14}C]valine forms the source of isotopic carbon may be considered to support the thesis that the valine carboxyl carbon forms a direct source of formate, since in both mammalian and microbial cells formate is not incorporated into uracil (Heinrich & Wilson, 1950; Edmunds, Delluva & Wilson, 1952).

Formic acid is known to participate in two stages in purine synthesis, providing a source from which $C_{(2)}$ and $C_{(8)}$ of the complete molecule are derived (e.g. Carter, 1956). Thus if the incorporation of ^{14}C

from [1-^{14}C]valine into adenine and guanine of normal cultures of *A. aerogenes* proceeds through the intermediary of formic acid it would be expected that the major part of the total activity of the purine molecule would be shared between $C_{(2)}$ and $C_{(8)}$. The finding that at least a quarter of the total activity of the guanine molecule is located at $C_{(2)}$ is therefore further evidence for the direct formation of metabolically active formic acid from the valine carboxyl group. In aminopterin-treated cultures of *A. aerogenes*, however, the accumulation of 5-amino-4-imidazolecarboxamide riboside as well as valine suggests that in the presence of the folic acid antagonist, $C_{(2)}$ and $C_{(8)}$ of the purine nucleus are derived from different sources.

Although it is uncertain whether the synthesis of the purine nucleotide from 5-amino-4-imidazolecarboxamide ribosyl phosphate occurs via inosinic acid or adenylosuccinic acid or both, the biosynthetic mechanisms leading to the formation of the imidazole derivative from glycine in certain cells seem well established (see, for example, Carter, 1956). It is particularly interesting that 5-amino-4-imidazolecarboxamide riboside (or ribotide) accumulates in the presence of aminopterin, since in the sequence of reactions leading to the formation of this compound the formylation of the intermediary glycinamide ribosyl phosphate is also dependent upon a folinic acid coenzyme (Goldthwait, Peabody & Greenberg, 1956*a*, *b*, *c*). It is possible, however, that the mechanism of synthesis of the imidazole intermediate in bacteria differs from that of avian and mammalian cells. Thus although Edmunds *et al.* (1951, 1952) and Koch (1955) found [2-^{14}C]glycine to give rise to [5-^{14}C]-guanine in yeast and *E. coli* respectively, Gots & Gollub (1956), in a study of 5-amino-4-imidazolecarboxamide ribotide synthesis by cell-free extracts of an *E. coli* mutant (B-96), were unable to demonstrate any requirement for glycine, formate, bicarbonate or aspartic acid.

The accumulation of the imidazole derivative by *A. aerogenes* is in accordance with previous findings in *E. coli* (Woolley & Pringle, 1950; Edwards *et al.* 1952), and the known inhibition by the folic acid antagonists of the incorporation of formate into nucleic acid purines (Skipper, Mitchell & Bennett, 1950; Goldthwait & Bendich, 1952). In the present system, however, there is an inhibition not only of the incorporation, but also of the production of formate. In this respect there seems to be some analogy between the accumulation of amino acids in the aminopterin-treated *A. aerogenes* cultures, and the depression of the synthesis of glycine from serine (a reaction which contributes to the formate 'pool') by folic acid deficiency in animal tissues (Elwyn & Sprinson, 1956).

The incorporation of formate into the methyl group of thymine, which in chick and rabbit bone-marrow cells is known to be inhibited by aminopterin (Totter & Best, 1955; Friedkin & Roberts, 1956), must involve a reductive step. Elwyn, Weissbach, Henry & Sprinson (1955) consider that two different types of folic acid coenzyme, in which the active intermediates are hydroxymethyl and formyl derivatives, are required for the introduction of the methyl group into thymine (and also into choline and methionine) and in the synthesis of purines respectively. Although these two forms of the coenzyme are interconvertible by a reversible di- (or tri-) phosphopyridine nucleotide enzyme, at least in the serine hydroxymethylase system (Kisluik & Sakami, 1955), studies in which the β-carbon atom of [$^{14}CD_2 \cdot OH$]serine has been shown to be transferred to the methyl group of thymine (Elwyn & Sprinson, 1950) and choline (Elwyn *et al.* 1955) without the loss of a deuterium atom, suggest that the rate of this interconversion is small compared with the rates of incorporation of the formyl and hydroxymethyl units. From these considerations the incorporation of ^{14}C from [1-^{14}C]-valine into thymine observed in the present work (Table 8) seems particularly high, although the value of approximately two for the ratio of the specific activity of adenine or guanine to that of thymine (Table 8) agrees with the figure given by Goldthwait & Bendich (1952) to express the ratio of formate incorporated into adenine and thymine by rat tissues.

The mechanism of the production of formate from the valine carboxyl group remains to be established experimentally. On the assumption that the accumulation of both alanine and valine is the result of the inhibition of the metabolism of the corresponding keto acids, it is possible that there is a common folinic acid-dependent step in the metabolism of both 3-methyl-2-oxobutanoic acid and pyruvic acid. It has been suggested by Kidder (1953), although without experimental justification, that a formyl derivative of folinic acid (CoF) may be a primary product of the dissimilation of pyruvate to acetyl-CoA and formic acid (e.g. Chantrenne & Lipmann, 1950). If this hypothesis is correct, as suggested by the recent observations of Chin, Krampitz & Novelli (1957) on the stimulation by tetrahydrofolic acid of the incorporation of formate into pyruvate by cell-free extracts of *E. coli*, a deficiency of CoF would be expected to lead to an inhibition of one pathway of pyruvate utilization. This would offer an explanation for both the reduced ability of folinic acid-deficient *Leuconostoc citrovorum* cells to decompose pyruvic and lactic acids (Chang, Silverman & Keresztesy, 1951), and the accumulation of alanine by *A. aerogenes* in the presence of aminopterin. The

mechanism of leucine biosynthesis suggested by T. Wainwright & D. D. Woods (personal communication) includes the formation of *iso*butyryl-CoA from 3-methyl-2-oxobutanoic acid, a reaction which is known to occur in the degradation of valine by extracts of rat heart and liver (Coon, 1955; Robinson, 1956; Robinson, Nagle, Bachhawat, Kupiecki & Coon, 1957). The cleavage of 3-methyl-2-oxobutanoic acid to *iso*butyryl-CoA and formic acid thus appears analogous to the cleavage of pyruvate to acetyl-CoA and formic acid, and it is possible that both of these reactions are dependent upon folinic acid.

SUMMARY

1. Throughout the period of inhibition of growth which follows the addition of the folic acid antagonist, aminopterin, to cultures of *Aerobacter aerogenes* in a simple chemically defined medium, alanine, valine and 5-amino-4-imidazolecarboxamide riboside accumulate in the medium.

2. Inhibition of growth of *A. aerogenes* by aminopterin is accompanied by a decrease in deoxyribonucleic acid content, which markedly exceeds that of any other cell component.

3. With the resumption of growth, which occurs after 15–18 hr. through the inactivation of the aminopterin, the accumulated intermediates are reutilized rapidly, and the chemical composition of the cells becomes similar to that of normal cells grown in the absence of the antagonist.

4. In the recovery cultures the accumulated alanine is utilized in the synthesis of a number of amino acids including leucine, for which it provides the α- and carboxyl-carbon atoms. The accumulated valine, however, is incorporated to a significant extent only into valine and leucine of the protein of *A. aerogenes*.

5. The synthesis of leucine from valine involves the elimination of the carboxyl group of the latter amino acid. In normal cultures of *A. aerogenes* this occurs with the formation of a C_1 unit other than carbon dioxide, which is utilized in the synthesis of the purine nucleus and the methyl group of thymine, and is probably formic acid.

6. An analogy is drawn between the cleavage of 3-methyl-2-oxobutanoic acid with the production of formic acid, and the dissimilation of pyruvate to acetyl-coenzyme A and formic acid, and it is suggested that both of these reactions may be dependent upon folinic acid and susceptible to inhibition by aminopterin.

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ADDENDUM

A Factorial Experiment to Study Factors which Reverse Aminopterin Inhibition

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The experiments reported in the preceding paper (Webb, 1958) showed that the inhibition of growth produced by aminopterin in cultures of *Aerobacter aerogenes* was partially prevented by the addition of glycine, adenine, L-threonine and L-glutamic acid to the culture medium. However, when attempts were made to investigate the significance of the individual components of this mixture, by omitting them one at a time from the culture medium, equivocal results were obtained. This appeared to be due to the marked stimulatory effect of L-glutamic acid on the growth of *A. aerogenes* in the simple medium, and to interactions between the effects of the remaining components. The effects of these substances were investigated therefore by means of a factorial experiment, such an experiment being ideally suited for the exploration of complex situations of this type.

METHODS

The culture conditions and details of the turbidity measurements are given by Webb (1958). Each of the four factors were employed at two levels, absent or present, giving the sixteen treatment combinations which are listed in Table 1. These mixtures were prepared in duplicate in 4 ml. of culture medium, and then inoculated with either an aminopterin-treated or untreated culture of *A. aerogenes*. This procedure was adopted in favour of that used in the growth experiments described in the main paper in order to overcome the variations in growth induced by the presence or absence of L-glutamic acid.

The tubes were incubated at 37° and the turbidity of each was measured at 0, 2·5, 5 and 21 hr. A replicate of the above procedure was carried out with a different overnight culture of *A. aerogenes* and different samples of aminopterin.

Mixtures. Mixtures were prepared in test tubes. When a factor was present in the treatment combination an amount sufficient to give a final concentration of mM was added in 1 ml. of the culture medium. All tubes were made up to a final volume of 4 ml. with the medium. Two sets of sixteen tubes, each corresponding to a separate treatment combination, were prepared in each replicate; numbers A1–A16 were inoculated from an aminopterin-treated culture, and numbers C1–C16 were inoculated from a control culture.

Aminopterin-treated culture. A volume (75 ml.) of medium was inoculated with an overnight culture of *A. aerogenes* and incubated at 37° until the turbidity reached an optical density of 0·40–0·45 (≡190–220 μg. of dry cells/ml.); 35 ml. of this culture was then transferred to a flask containing 5 ml. of a 1:1000 solution of aminopterin at 37° and 2 ml. of this final mixture was added to each of the tubes A1–A16, giving a final aminopterin concentration of 1:24 000 (≏0·1 mM).

Control culture. A volume (35 ml.) of the culture was similarly treated with 5 ml. of medium and 2 ml. samples from this mixture were added to tubes C1–C16.

EXPERIMENTAL DESIGN

An examination of the method by which the organisms from the overnight culture were allotted to the treatments shows that two stages were involved. In the first stage aminopterin-treated and control cultures were prepared; then, in the second stage, these cultures were each used to inoculate the appropriate set of sixteen tubes containing the mixtures under study. Thus the experiment is of the split-plot type; the aminopterin-treated and control cultures may be considered whole-plots, and each contains 16 split-plots arranged in a 2^4 factorial scheme. A discussion of this design and its statistical analysis is given in many textbooks on the design of experiments (e.g. Kempthorne, 1952).

RESULTS

Results obtained after incubation for 5 hr. are shown in Table 1, and the analysis of variance is given in Table 2. The analysis for the split-plot design is in two parts with separate error terms: the whole-plot analysis, which compares the overall difference between the control and aminopterin-treated cultures (error *a*), and the split-plot analysis, which compares the effects of the factorial treatments within each whole-plot (error *b*). A substantial difference was obtained between the overall effects of the control and aminopterin-treated cultures. The experimental design used, however, does not allow an effective estimate of the error of this comparison to be made since it is based on only 1 degree of freedom. Thus no useful test of significance of the whole-plot effect is possible. Nevertheless this does not matter since inhibition of growth by aminopterin is well established.

In the split-plot analysis there are 15 degrees of freedom, which allow an examination to be made of the main effects and all the interactions of the sixteen factorially arranged treatments. Moreover, a further 15 degrees of freedom allow an examination to be made of the interaction of the effects of the whole-plot and split-plot treatments, i.e. an examination can be made of whether or not the effects of the sixteen factorial treatments are identical in the aminopterin-treated and control cultures. All of these thirty components are investigated with equal precision and their mean squares are tested for significance against error *b*. In the full analysis all of these individual mean squares were computed, but, for brevity, only the main effects and significant interactions are shown in Table 2.

It can be seen that the effects of adenine and L-glutamic acid are highly significant, and the effects of glycine and L-threonine are not significant. Further, the adenine × aminopterin (BE), L-glutamic acid × aminopterin (DE) and adenine × L-glutamic acid × aminopterin (BDE) interactions are all highly significant, whereas all other interactions are not significant at the $P = 0{\cdot}05$ level. If the experiment is analysed in two independent parts corresponding to the control and aminopterin-treated whole-plots it is found that in both the adenine × L-glutamic acid interactions are significant. Since these interactions are in opposite directions, however, the same interaction in the overall analysis is not significant.

The above analysis indicates that glycine and L-threonine have no demonstrable influence on the system and that the effects of adenine and L-glutamic acid depend on whether or not aminopterin is also present in the culture medium. Fig. 1 demonstrates graphically the BDE three-way

Table 1. *Growth of* Aerobacter aerogenes *in aminopterin-treated and control media with and without glycine, adenine,* L-*threonine and* L-*glutamic acid*

The turbidity readings (optical density) of cultures after incubation for 5 hr. at 37°, after addition of various combinations of glycine, adenine, L-threonine and L-glutamic acid in aminopterin-treated and control media. +, The component was added to give a final concentration of mM; −, the component was omitted. The experiment was carried out in two replicates, with different samples of aminopterin.

Tube no.	Glycine	Adenine	L-Threonine	L-Glutamic acid	Turbidity (*E*) I	Turbidity (*E*) II
			Aminopterin-treated series			
A1	−	−	−	−	0·209	0·150
2	+	−	−	−	0·221	0·166
3	−	+	−	−	0·366	0·184
4	+	+	−	−	0·351	0·198
5	−	−	+	−	0·244	0·188
6	+	−	+	−	0·212	0·173
7	−	+	+	−	0·376	0·219
8	+	+	+	−	0·402	0·228
9	−	−	−	+	0·188	0·176
10	+	−	−	+	0·221	0·191
11	−	+	−	+	0·460	0·287
12	+	+	−	+	0·544	0·313
13	−	−	+	+	0·241	0·200
14	+	−	+	+	0·254	0·186
15	−	+	+	+	0·533	0·352
16	+	+	+	+	0·535	0·374
			Control series			
C1	−	−	−	−	0·630	0·718
2	+	−	−	−	0·653	0·752
3	−	+	−	−	0·641	0·758
4	+	+	−	−	0·622	0·730
5	−	−	+	−	0·648	0·732
6	+	−	+	−	0·678	0·744
7	−	+	+	−	0·631	0·738
8	+	+	+	−	0·631	0·722
9	−	−	−	+	0·896	0·892
10	+	−	−	+	0·918	0·886
11	−	+	−	+	0·828	0·832
12	+	+	−	+	0·823	0·842
13	−	−	+	+	0·899	0·877
14	+	−	+	+	0·884	0·897
15	−	+	+	+	0·822	0·818
16	+	+	+	+	0·816	0·837

Table 2. *Analysis of variance of the data shown in Table* 1

Levels of significance are given in parentheses in the last column.

Source of variation	Degrees of freedom	Mean square
A. Whole-plot analysis		
Between replicates	1	0·0162
Control v. aminopterin-treated cultures (E)	1	3·9268
Error (*a*)	1	0·0998
B. Split-plot analysis		
Factorial treatments:	(15)	
Glycine (A)	1	0·0011
Adenine (B)	1	0·0558 ($P<0·001$)
L-Threonine (C)	1	0·0031
L-Glutamic acid (D)	1	0·2385 ($P<0·001$)
Interactions	11	0·0006
Whole-plot × split-plot treatments, interactions:	(15)	
BE	1	0·1516 ($P<0·001$)
DE	1	0·0386 ($P<0·001$)
BDE	1	0·0319 ($P<0·001$)
Remainder	12	0·0008
Error (*b*)	30	0·00194

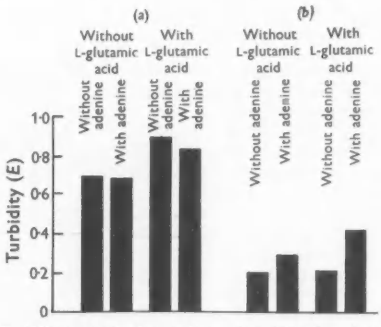

Fig. 1. Effect of adenine, L-glutamic acid and aminopterin on the growth of *A. aerogenes* after incubation for 5 hr. Values are the means of the BDE three-way table. (*a*) Without aminopterin; (*b*) with aminopterin.

table. It can be seen that aminopterin depresses growth under all conditions. Also the addition of L-glutamic acid causes greater growth, but the magnitude of this effect depends considerably on the absence or presence of the other two substances. The effect of adenine, however, is opposite in the aminopterin-treated and control cultures; in the control cultures the adenine depresses the stimulating effect of L-glutamic acid, whereas in the aminopterin-treated cultures the addition of adenine enhances growth even when glutamic acid is not present in the medium. If L-glutamic acid is present, however, the enhancement of growth is greater still.

Similar results were obtained after incubation for 2·5 and 21 hr. and are not presented in this paper since they add little extra information to the above results.

SUMMARY

1. Glycine and L-threonine have no effect on the growth of *Aerobacter aerogenes* when they are added to aminopterin-treated or control media.

2. L-Glutamic acid stimulates growth in both aminopterin-treated and control media.

3. Adenine stimulates growth in aminopterin-treated media and in control media inhibits the effect of L-glutamic acid.

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Regulation of Glucose Uptake by Muscle

1. THE EFFECTS OF INSULIN, ANAEROBIOSIS AND CELL POISONS ON THE UPTAKE OF GLUCOSE AND RELEASE OF POTASSIUM BY ISOLATED RAT DIAPHRAGM

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Gemmill & Hamman (1941) showed that the uptake of glucose by isolated rat diaphragm is enhanced by the presence of insulin *in vitro*. The factors which regulate the uptake of glucose by this tissue and the mechanism of this action of insulin are largely unknown, though it is thought that insulin may increase glucose uptake by accelerating the entry of glucose into the muscle cell (Levine & Goldstein, 1955; Park, Bornstein & Post, 1955; Park & Johnson, 1955). It is known that the uptake of glucose by many cells and tissues is greater under conditions where oxidative phosphorylation is impaired, e.g. under anaerobic conditions. It was of interest therefore to investigate the effects of anaerobiosis and of substances which inhibit oxidative phosphorylation on the uptake of glucose by diaphragm and to compare their effects with those of insulin.

In preliminary communications (Randle, 1956; Randle & Smith, 1957) we have noted that the uptake of glucose by isolated diaphragm in a medium buffered with bicarbonate is increased by oxygen lack and by 2:4-dinitrophenol, sodium arsenite or sodium cyanide. On the other hand, when diaphragm is incubated in a medium buffered with phosphate uptake of glucose is apparently not increased by oxygen lack (Walaas & Walaas, 1952; Demis & Rothstein, 1954) or by 2:4-dinitrophenol (Villee, Deane & Hastings, 1949; Pierce & Field, 1949). Consequently, it was important to define more clearly the conditions under which oxygen lack and substances which inhibit oxidative phosphorylation will stimulate uptake of glucose by diaphragm.

Calkins, Taylor & Hastings (1954) and Creese (1954) have shown that potassium is lost from the muscle when isolated rat diaphragm is incubated in a bicarbonate medium under anaerobic conditions. Because of the possibility that loss of potassium from isolated diaphragm might be in some way associated with glucose uptake, we have compared the uptake of glucose with the loss of potassium under anaerobic conditions, in the presence of insulin and in the presence of substances which inhibit oxidative phosphorylation.

METHODS AND PROCEDURE

Incubation media. The composition of the basal media used is given in Table 1. A.R.-grade chemicals were used if commercially available.

Insulin. Crystalline insulin (Boots Pure Drug Co. Ltd. or Novo Terapeutisk Laboratories) was dissolved in N/300-HCl at a concentration of 20 units/ml. This stock solution was diluted with medium to the required concentration (usually 0·1 unit/ml.) just before each experiment.

Animals. Diaphragm muscle was obtained from male albino Wistar rats of 100–150 g. wt., fed with a stock laboratory diet (Bruce & Parkes, 1949). Food was withheld from intact rats for 18–20 hr. before the experiment; hypophysectomized or adrenalectomized rats, which were used 8–10 days after operation, were not starved. The animals had free access to water (or 1% NaCl for adrenalectomized rats) at all times.

Procedure. Rats were killed by decapitation and exsanguinated. Diaphragms were then rapidly removed, and

Table 1. *Composition of basal media used for incubation of isolated diaphragm*

Media containing bicarbonate were gassed with $O_2 + CO_2$ or $N_2 + CO_2$ (95:5); pH 7·4 at 37°. Tris medium and phosphate medium were gassed with O_2 or N_2; pH 7·4 at 37°. D-Glucose was present in all media (2·5 mg./ml.); this is included in the calculated osmolarity (the units m-osmoles have been suggested and defined by Gamble, 1950).

Medium	Composition (m-equiv./l.)									Calc. osmolarity (m-osmoles/l.)
	Na	K	Ca	Mg	Tris*	Cl	HCO_3	PO_4†	SO_4	
Bicarbonate‡	124·8	5·2	2·7	2·6	—	105·7	27·2	1·8	0·6	281
High phosphate–bicarbonate	133·0	5·2	2·7	2·6	—	77·9	27·2	37·8	0·6	281
Tris*	108·8	5·2	2·7	2·6	16·0	132·9	—	1·8	0·6	281
Phosphate	143·2	5·1	2·7	2·6	—	132·8	—	18·2	0·6	309

* 2-Amino-2-hydroxymethylpropane-1:3-diol.
† Assuming valency of 1·8 at pH 7·4.
‡ Gey & Gey (1936).

spread on filter paper (Whatman no. 40), and the posterior segment was removed and the remainder divided along the central tendon to yield two hemidiaphragms. These were then soaked for 5 min. at room temperature in the appropriate basal medium (previously saturated with the appropriate gas or gas mixture). They were then lightly blotted and transferred to flasks for incubation. In most experiments each hemidiaphragm was incubated in 1 ml. of medium in a 20 ml. conical flask, though in certain experiments three to eight hemidiaphragms were incubated together in 3–12 ml. of medium in a 50 ml. conical flask. The flasks were then gassed with the appropriate gas or gas mixture, and sealed with rubber bungs and incubated with shaking in a water bath at 37°. After incubation, which was usually for 1 hr., the flasks were cooled, and samples of medium taken for analysis, and the hemidiaphragms were removed, blotted with filter paper and weighed on a torsion balance. In experiments where the course of glucose uptake or potassium release was followed five to eight hemidiaphragms were incubated together in 5–12 ml. of medium in a 50 ml. conical flask fitted with a side arm and stopcock. The gas phase was then renewed or altered when samples of medium were taken during incubation.

Analytical methods

pH. This was determined with the glass electrode.

Glucose uptake. This was determined by measuring the disappearance of glucose from the medium. The glucose content of the medium was determined before and after each incubation by one or more of three methods: (i) colorimetrically by the Somogyi (1945) modification of Nelson's method, (ii) manometrically with glucose oxidase (Sigma Chemical Co., obtained from George T. Gurr Ltd.) after the method of Keilin & Hartree (1948) and (iii) colorimetrically with glucose oxidase (Sigma Chemical Co.), peroxidase (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) and *o*-dianisidine after the method of Huggett & Nixon (1957).

In some experiments the disappearance of glucose from the medium was followed with [^{14}C]glucose. [$^{14}C_6$]Glucose (15 mc/m-mole; the Radiochemical Centre, Amersham, Bucks) was added to the medium at 0·6 μc/ml. After incubation, samples of medium were desalted by addition of ethanol to 70% (v/v) and the resulting supernatant was dried *in vacuo*. The residues were taken up in a 1% (w/v) solution of unlabelled glucose and samples (5 μl.) were chromatographed on Whatman no. 1 paper with the pyridine–ethyl acetate–water (1:2:2) system of Jermyn & Isherwood (1949). The spots were located by markers, and cut out, and the glucose was eluted on to aluminium planchets, and dried under an infrared lamp and counted beneath a thin end-window Geiger–Müller tube. Individual spots gave from 700 to 1200 counts/min.; at least 1000 counts were recorded for each sample.

Potassium loss. Loss of potassium from the diaphragm was calculated from the initial and final potassium contents of the incubation medium. Potassium was estimated with an EEL flame photometer, 1:50 dilutions of medium being compared with standard solutions containing 0·1 or 0·2 m-equiv. of potassium/l.

Statistical analysis. The significance of differences between means has been established by calculating t; P, the probability of differences being due to chance, was obtained from tables for t (Fisher & Yates, 1943).

Table 2. *Uptake of glucose by isolated rat diaphragm under aerobic or anaerobic conditions in media buffered with bicarbonate, phosphate or tris*

Composition of media is given in Table 1. The number of observations is given in parentheses. Glucose analyses were by the colorimetric method of Somogyi (1945).

Rats	Medium	Gas phase: Aerobic	Gas phase: Anaerobic	Initial pH	Glucose uptake. Mean ± S.E.M. (mg. of glucose/g. of wet diaphragm/hr. of incubation): Aerobic	Glucose uptake. Mean ± S.E.M. (mg. of glucose/g. of wet diaphragm/hr. of incubation): Anaerobic	Difference ± s.e. of difference	Significance of difference (P)
Intact	Bicarbonate	O_2+CO_2	N_2+CO_2	7·4	2·6 ± 0·07 (53)	5·6 ± 0·09 (53)	3·0 ± 0·12	<0·001
	Phosphate	O_2	N_2	7·4	2·9 ± 0·12 (36)	3·2 ± 0·10 (36)	0·3 ± 0·15	<0·05 > 0·02
	Phosphate	O_2+CO_2	N_2+CO_2	6·9	2·5 ± 0·18 (12)	3·5 ± 0·11 (12)	1·0 ± 0·21	<0·001
	Phosphate	O_2	N_2	6·9	2·2 ± 0·20 (24)	3·4 ± 0·13 (24)	1·2 ± 0·24	<0·001
	Tris	O_2	N_2	7·4	2·4 ± 0·24 (6)	2·6 ± 0·22 (6)	0·2 ± 0·33	<0·6 > 0·5
Hypophysectomized	Bicarbonate	O_2+CO_2	N_2+CO_2	7·4	3·6 ± 0·06 (15)	5·5 ± 0·13 (15)	1·9 ± 0·14	<0·001
Adrenalectomized	Bicarbonate	O_2+CO_2	N_2+CO_2	7·4	3·0 ± 0·27 (5)	4·5 ± 0·36 (5)	1·5 ± 0·45	<0·02 > 0·01

RESULTS

Glucose uptake under anaerobic conditions

In a medium buffered with bicarbonate the uptake of glucose by isolated rat diaphragm is greater under anaerobic than under aerobic conditions (Tables 2 and 3). This has been demonstrated with methods of glucose analysis based on copper reduction (Somogyi, 1945), glucose oxidase (Keilin & Hartree, 1948) or [^{14}C]glucose (Tables 2 and 3). This effect of anaerobiosis on glucose uptake is seen with diaphragms from intact, hypophysectomized or adrenalectomized rats (Table 2). On the other hand, if diaphragm is incubated in a medium buffered with phosphate (initial pH 7·4) the anaerobic–aerobic difference in glucose uptake is not significant (Table 2; Walaas & Walaas, 1952; Demis & Rothstein, 1954).

Further experiments have been carried out in an attempt to explain this difference in response with the two media. The composition of phosphate-buffered medium differs from that of bicarbonate-buffered medium in the following respects: (i) its calculated osmotic pressure is slightly higher; (ii) it does not contain bicarbonate; (iii) it contains considerably more phosphate (Table 1). The influence of each of these differences in composition has been studied separately. The calculated osmotic pressure of phosphate medium was first adjusted to that of bicarbonate medium by omission of the appropriate amount of sodium chloride. This change did not affect the lack of response to anaerobiosis in phosphate medium (Table 4). To determine the effect of phosphate concentration, the phosphate content of bicarbonate medium was increased to more than twice that of phosphate medium, the calculated osmolarity being kept constant by appropriate reduction of the sodium chloride content (high phosphate–bicarbonate medium, Table 1). The uptakes of glucose under aerobic or anaerobic conditions and the anaerobic − aerobic difference were all enhanced in high phosphate–bicarbonate medium (Table 5). Furthermore, when diaphragm was incubated in a medium buffered with 2-amino-2-hydroxymethylpropane-1:3-diol(tris) which, though lacking bicarbonate, contained only the same amount of phosphate as bicarbonate-buffered medium (Table 1), glucose uptake was not augmented under anaerobic conditions (Table 2). In an attempt to determine whether the bicarbonate ion had a specific effect (as opposed to its buffering activity) phosphate-buffered medium was gassed with $O_2 + CO_2$ or $N_2 + CO_2$ (95:5) instead of with O_2 or N_2. Under these conditions uptake of glucose by the diaphragm was enhanced in phosphate medium under anaerobic conditions (Table 2); but it was noted that the initial pH of the medium fell from 7·4 to 6·9 when gassed with mixtures containing 5% of CO_2. When phosphate medium, of initial pH 6·9 and gassed with pure N_2 or O_2, was used, a similar anaerobic-aerobic difference was observed (Table 2). It seems likely, therefore, that the stimulating effect of anaerobiosis on glucose uptake in phosphate medium gassed with mixtures containing 5% of CO_2 is related to the fall in the initial pH of the medium rather than to the bicarbonate which is thereby introduced.

The response to anaerobiosis of the glucose uptake of isolated diaphragm appears to be related to the change of pH which occurs in the medium

Table 3. *Uptake of glucose by isolated rat diaphragm under aerobic or anaerobic conditions in bicarbonate medium*

Each figure is the mean of two values, obtained from the incubation of five hemidiaphragms in 3 ml. of bicarbonate medium. Glucose analyses were by the glucose oxidase method (Keilin & Hartree, 1948).

Expt. no.	Glucose uptake (mg. of glucose/g. of wet diaphragm/hr. of incubation)	
	Aerobic	Anaerobic
1	3·5	5·1
2	3·0	6·1
	Uptake of [$^{14}C_6$]glucose (counts/min. in glucose/g. of wet diaphragm/hr. of incubation)	
3	101 000	394 000
4	120 000	588 000

Table 4. *Uptake of glucose by isolated rat diaphragm under aerobic or anaerobic conditions in phosphate media of differing osmolarity (initial* pH 7·4)

Number of observations is given in parentheses. Glucose analyses were by the colorimetric method of Somogyi (1945).

Calc. osmolarity (m-osmoles/l.)	Glucose uptake. Mean ± S.E.M. (mg. of glucose/g. of wet diaphragm/hr. of incubation)		Difference ± S.E. of difference
	Aerobic	Anaerobic	
281*	2·2 ± 0·18 (6)	2·7 ± 0·10 (6)	0·5 ± 0·21
309†	2·4 ± 0·23 (6)	2·8 ± 0·36 (6)	0·4 ± 0·43

* Osmolarity was reduced by omission of 14 m-equiv. of NaCl/l.

† Phosphate medium as in Table 1.

Table 5. *Influence of phosphate content of bicarbonate medium on uptake of glucose by isolated rat diaphragm under aerobic or anaerobic conditions*

Composition of media is given in Table 1. Number of observations is given in parentheses. Glucose analyses were by the colorimetric method of Somogyi (1945).

Medium	Glucose uptake. Mean ± S.E.M. (mg. of glucose/g. of wet diaphragm/hr. of incubation)		Difference ± S.E. of difference (anaerobic – aerobic)	Significance of difference (*P*)
	Aerobic	Anaerobic		
Bicarbonate	2·2 ± 0·12 (12)	4·5 ± 0·12 (12)	2·3 ± 0·18	<0·001
High phosphate–bicarbonate	2·8 ± 0·14 (12)	7·0 ± 0·28 (12)	4·2 ± 0·29	<0·001
Difference ± S.E. of difference (high phosphate–bicarbonate – bicarbonate)	0·6 ± 0·18	2·5 ± 0·28	1·9 ± 0·34	—
Significance of difference (*P*)	<0·02 <0·01	<0·001	<0·001	—

Table 6. *Fall in* pH *of medium during incubation of isolated diaphragm under aerobic or anaerobic conditions*

Composition of media is given in Table 1. Number of observations is given in parentheses.

Medium	Initial pH	pH fall during incubation. Mean ± S.E.M. (pH units/hr.)	
		Aerobic	Anaerobic
Bicarbonate	7·4	0·11 ± 0·15 (4)	0·11 ± 0·15 (4)
Phosphate	7·4	0·66 ± 0·08 (4)	0·60 ± 0·13 (4)
Phosphate	6·9	0·07 ± 0·14 (4)	0·35 ± 0·16 (4)
Tris	7·4	0·55 (1)	0·80 (1)

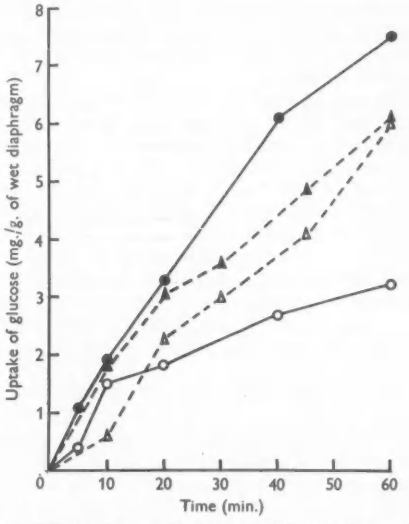

Fig. 1. Effect of anaerobiosis on uptake of glucose by diaphragm. Incubations were made in bicarbonate medium under the following conditions: ●, anaerobic for 60 min.; ○, aerobic for 60 min.; ▲, anaerobic for 20 min. then aerobic for 40 min.; △, aerobic for 20 min. then anaerobic for 40 min.

during incubation. With bicarbonate medium little change of pH is evident after incubation for 1 hr. under either aerobic or anaerobic conditions and glucose uptake is increased under anaerobic conditions. In media buffered with phosphate or tris (of initial pH 7·4) the pH falls substantially during incubation for 1 hr. and there is no anaerobic–aerobic difference either in glucose uptake or in the fall of pH. On the other hand, in phosphate medium of initial pH 6·9 glucose uptake is enhanced under anaerobic conditions and the pH change after incubation for 1 hr. is much less (Tables 2 and 6). A similar relation between initial pH and fall of pH during aerobic incubation of diaphragm in phosphate medium was observed by Stadie & Zapp (1947).

The course of glucose uptake by diaphragm in bicarbonate medium under aerobic or anaerobic conditions is shown in Fig. 1. An enhanced rate of glucose uptake under anaerobic conditions is evident at all times during incubation for 1 hr. and is detectable within 5 min. of commencing incubation. If aerobic conditions are established after a period of anaerobiosis the rate of glucose uptake falls; conversely, if anaerobic conditions are established after an initial aerobic incubation the rate of glucose uptake increases (Fig. 1). To demonstrate the statistical significance of these observations a number of hemidiaphragms were incubated (i) for 1 hr. under aerobic conditions; (ii) for 1 hr. under anaerobic conditions; (iii) for 20 min. under aerobic conditions followed by 40 min. under anaerobic conditions; (iv) for 20 min. under anaerobic conditions followed by 40 min. under aerobic conditions. The uptake of glucose was measured after a total incubation of 1 hr. in each

Table 7. *Uptake of glucose and release of potassium by isolated diaphragm under aerobic or anaerobic conditions in bicarbonate medium*

Number of observations is given in parenthesis. Glucose analyses were by the colorimetric method of Somogyi (1945). *P* is not greater than 0·01 for differences between means, except for (iii) – (ii) where *P* is > 0·05 for both glucose uptake and potassium release.

Conditions during incubation for 1 hr.	Glucose uptake. Mean ± S.E.M. (mg. of glucose/g. of wet diaphragm/hr. of incubation)	Potassium release. Mean ± S.E.M. (μ-equiv. of potassium/g. of wet diaphragm/hr. of incubation)
(i) Anaerobic	5·2 ± 0·24 (18)	37 ± 1·6 (12)
(ii) Anaerobic for 20 min. then aerobic for 40 min.	4·2 ± 0·16 (18)	21 ± 0·7 (12)
(iii) Aerobic for 20 min. then anaerobic for 40 min.	3·9 ± 0·11 (18)	18 ± 1·8 (12)
(iv) Aerobic	2·3 ± 0·10 (18)	6 ± 1·3 (12)

Table 8. *Glucose utilization in medium during incubation*

First incubation: 12 hemidiaphragms in 12 ml. of bicarbonate medium were incubated for 30 min. Second incubation: 1 ml. samples of medium from first incubation were incubated alone for a further 60 min. under the same conditions.

Conditions	Change in glucose content of medium (mg./ml./hr.) First incubation	Second incubation
Aerobic	−0·40	+0·07
Anaerobic	−0·92	−0·04
Aerobic without tissue	0·00	−0·02

case. It was found that conditions which were partly aerobic and partly anaerobic (ii, iii) were associated with a significantly greater uptake of glucose than conditions which were wholly aerobic (iv), and with a significantly smaller uptake than conditions which were wholly anaerobic (i) (Table 7). These experiments show that the changes which occur in isolated diaphragm under anaerobic conditions and which lead to an increased uptake of glucose can be reversed if aerobic conditions are restored.

Beloff-Chain *et al.* (1953) found that phosphoglucomutase readily diffuses from diaphragm and Zierler, Levy & Andres (1953) claim that enzymes capable of dissimilating glucose 1-phosphate or fructose 1:6-diphosphate to lactic acid may leak from diaphragm during incubation in phosphate medium. On the other hand, Shaw & Stadie (1957) believe that aldolase is the only glycolytic enzyme to do so. Nevertheless, it was conceivable that some of the glucose which disappeared from the medium during incubation of diaphragm under our conditions was being utilized as a result of the action of enzymes which had leaked into the medium. In order to examine this possibility, medium in which diaphragms had been incubated for 30 min., either under aerobic or under anaerobic conditions, was incubated by itself for a further period of 1 hr. In neither case did any more glucose disappear from the medium after the tissue had been removed (Table 8).

Goldstein, Mullick, Huddlestun & Levine (1953) believe that a humoral substance may be released from muscle, during muscular contraction *in vivo*, which is capable of facilitating the entry of some sugars into the cells of other resting muscles. The possibility that a substance might be released from diaphragm during incubation under anaerobic conditions which could promote glucose uptake was therefore tested experimentally. Eight hemidiaphragms were incubated in 6 ml. of bicarbonate medium for 1 hr. under either (*a*) aerobic or (*b*) anaerobic conditions. Glucose uptake (by the method of Somogyi, 1945) was (*a*) 2·16 and (*b*) 3·93 mg./g. of wet diaphragm/hr. With each resultant medium the glucose content was readjusted to 2·5 mg./ml. and an equal volume of fresh bicarbonate medium added. Fresh hemidiaphragms were then incubated singly for 1 hr. under aerobic conditions in six 1 ml. portions of each of these two new media: glucose uptake in the second incubation was: (*a*) 2·4 ± 0·23 and (*b*) 2·0 ± 0·14 mg./g. of wet diaphragm/hr. (mean ± S.E.M.). No stimulation of glucose uptake had therefore occurred with the medium from anaerobic incubation.

Influence of some cell poisons on uptake of glucose

The uptake of glucose by isolated diaphragm incubated under aerobic conditions in bicarbonate medium is increased in the presence of 2:4-dinitrophenol (50 μM or 0·25 mM), sodium arsenite (mM), sodium arsenate (10 mM), sodium cyanide (mM) or sodium salicylate (5 mM) (Table 9). Sodium azide (mM or 10 mM) was without effect on glucose uptake. Sodium *p*-hydroxybenzoate (5 mM),

Table 9. *Influence of cell poisons on aerobic uptake of glucose by isolated rat diaphragm*

Number of observations is given in parentheses. Glucose analyses were by the colorimetric method of Somogyi (1945).

Expt. no.	Medium	Addition	Glucose uptake. Mean ± S.E.M. (mg. of glucose/g. of wet diaphragm/hr. of incubation)	Difference ± S.E. of difference	Significance of difference (*P*)
1	Bicarbonate	None (control)	2·0 ± 0·15 (12)	—	—
		2:4-Dinitrophenol (50 μM)	3·1 ± 0·15 (6)	1·1 ± 0·21	<0·001
		(0·25 mM)	5·0 ± 0·32 (6)	3·0 ± 0·35	<0·001
		Sodium arsenite (mM)	6·8 ± 0·33 (12)	4·8 ± 0·37	<0·001
		Sodium cyanide (mM)	8·4 ± 0·36 (12)	6·4 ± 0·39	<0·001
2	Bicarbonate	None (control)	2·5 ± 0·20 (12)	—	—
		Sodium salicylate (5 mM)	4·0 ± 0·28 (12)	1·5 ± 0·35	<0·01
		Sodium *p*-hydroxybenzoate (5 mM)	2·0 ± 0·21 (6)	−0·5 ± 0·29	<0·2>0·1
3	Bicarbonate	None (control)	2·6 ± 0·24 (6)	—	—
		Sodium arsenate (mM)	3·2 ± 0·18 (6)	0·6 ± 0·30	<0·1>0·05
		Sodium azide (mM)	3·0 ± 0·14 (6)	0·4 ± 0·28	<0·2>0·1
4	Bicarbonate	None (control)	3·2 ± 0·51 (6)	—	—
		Sodium arsenate (10 mM)	6·1 ± 0·51 (6)	2·9 ± 0·72	<0·01>0·001
		Sodium azide (10 mM)	2·1 ± 0·35 (6)	−1·1 ± 0·62	<0·2>0·1
5	Phosphate (pH 7·4)	None (control)	3·2 ± 0·28 (6)	—	—
		2:4-Dinitrophenol (0·25 mM)	2·6 ± 0·35 (6)	−0·6 ± 0·44	<0·3>0·2
		Sodium salicylate (5 mM)	2·8 ± 0·13 (6)	−0·4 ± 0·37	<0·3>0·2
		Sodium cyanide (mM)	3·6 ± 0·39 (6)	0·4 ± 0·41	<0·4>0·3

Table 10. *Influence of anaerobiosis on the response to insulin of isolated diaphragm in bicarbonate medium*

Number of observations is given in parentheses. Glucose analyses were by the colorimetric method of Somogyi (1945).

Expt. no.	Concn. of insulin (unit/ml.)	Glucose uptake. Mean ± S.E.M. (mg. of glucose/g. of wet diaphragm/hr. of incubation)		Difference ± S.E. of difference	Significance of difference (*P*)
		Aerobic	Anaerobic		
1	0	3·7 ± 0·31 (6)	6·4 ± 0·44 (6)	2·7 ± 0·54	<0·001
	10^{-3}	8·0 ± 0·62 (6)	6·9 ± 0·24 (6)	−1·1 ± 0·66	<0·2>0·1
	Difference ± S.E. of difference	4·3 ± 0·7	0·5 ± 0·5	—	—
	Significance of difference (*P*)	<0·001	<0·4>0·3	—	—
2	0	2·3 ± 0·17 (6)	6·6 ± 0·31 (6)	4·3 ± 0·35	<0·001
	0·1	9·1 ± 0·24 (6)	8·7 ± 0·24 (6)	−0·4 ± 0·34	<0·4>0·3
	Difference ± S.E. of difference	6·8 ± 0·29	2·1 ± 0·39	—	—
	Significance of difference (*P*)	<0·001	<0·001	—	—

Table 11. *Influence of cell poisons on the response to insulin of isolated diaphragm in bicarbonate medium*

Number of observations is given in parentheses. Glucose analyses were by the method of Huggett & Nixon (1957).

Cell poison	Glucose uptake. Mean ± S.E.M. (mg. of glucose/g. of wet diaphragm/hr. of incubation)		
	Insulin (0·1 unit/ml.) alone (*A*)	Cell poison alone (*B*)	Insulin (0·1 units/ml.) + cell poison (*C*)
2:4-Dinitrophenol (0·25 mM)	11·6 ± 0·44 (6)	5·0 ± 0·34 (6)	6·0 ± 0·42 (6)
	P; for $(B-A)$ <0·001; for $(C-A)$ <0·001; for $(C-B)$ <0·2>0·1		
2:4-Dinitrophenol (0·25 mM)	—	3·3 ± 0·17 (14)	4·3 ± 0·26 (14)
	P; for $(C-B)$ <0·01>0·001		
Sodium arsenite (mM)	11·8 ± 0·45 (10)	8·4 ± 0·36 (10)	9·5 ± 0·25 (10)
	P; for $(B-A)$ <0·001; for $(C-A)$ <0·001; for $(C-B)$ <0·05>0·01		
Sodium cyanide (mM)	10·8 ± 0·42 (10)	8·8 ± 0·56 (10)	12·8 ± 0·44 (10)
	P; for $(B-A)$ <0·02>0·05; for $(C-A)$ <0·01; for $(C-B)$ <0·001		
Sodium salicylate (5 mM)	11·3 ± 0·36 (6)	5·4 ± 0·31 (6)	7·8 ± 0·48 (6)
	P; for $(B-A)$ <0·001; for $(C-A)$ <0·001; for $(C-B)$ <0·01>0·001		

Table 12. *Comparison of the effect of anaerobiosis, cell poisons or insulin on uptake of glucose and loss of potassium by isolated rat diaphragm*

Where P is not given it is not greater than 0·01. Number of observations is given in parentheses. Glucose analyses were by the colorimetric method of Somogyi (1945). Figures for glucose uptake were calculated from data given in Table 11.

Expt. no.	Medium	Addition	Potassium loss. Mean ± S.E.M. (μ-equiv. of potassium/g. of wet diaphragm/hr. of incubation)	Glucose uptake. Mean ± S.E.M. (μ-equiv. of glucose/g. of wet diaphragm/hr. of incubation)	Difference ± S.E. of difference	
					Potassium	Glucose
1	Bicarbonate	None (aerobic control)	8±0·9 (6)	13±1·0 (6)	—	—
		Insulin (0·1 unit/ml.)	5±0·9 (6)	51±1·3 (6)	−3±1·3†	38±1·6
2	Bicarbonate	None (aerobic control)	7±1·3 (12)	13±0·8 (12)	—	—
		None (anaerobic)	37±1·6 (12)	28±1·7 (12)	30±2·0	15±1·8
3	Bicarbonate	None (aerobic control)	14±1·3 (12)	11±0·8 (12)	—	—
		2:4-Dinitrophenol (50 μM)	44±1·8 (6)	17±0·8 (6)	30±2·2	6±1·1
		(0·25 mM)	47±1·5 (6)	28±1·8 (6)	33±2·0	17±2·0
		Sodium arsenite (mM)	37±2·7 (12)	38±1·8 (12)	23±2·7	27±2·0
		Sodium cyanide (mM)	33±2·1 (12)	47±2·0 (12)	19±2·4	36±2·2
4	Bicarbonate	None (aerobic control)	8±1·2 (14)	14±1·1 (14)	—	—
		Sodium salicylate (5 mM)	31±1·5 (14)	22±1·3 (14)	23±1·9	8±1·6
		Sodium *p*-hydroxybenzoate (5 mM)	8±0·9 (6)	11±1·2 (6)	0	−3±1·6*
5	Bicarbonate	None (aerobic control)	12±2·3 (6)	16±3·4 (6)	—	—
		Sodium arsenate (10 mM)	28±4·1 (6)	34±3·4 (6)	16±4·7	18±4·8
		Sodium azide (10 mM)	48±2·4 (6)	12±1·4 (6)	36±3·3	−4±3·3*
6	Phosphate (pH 7·4)	None (aerobic control)	13±2·1 (6)	15±1·9 (6)	—	—
		None (anaerobic)	44±1·8 (6)	21±1·1 (6)	31±2·8	6±2·2†
7	Phosphate (pH 7·4)	None (aerobic control)	17±0·6 (6)	18±1·6 (6)	—	—
		2:4-Dinitrophenol (0·25 mM)	57±2·3 (6)	14±1·8 (6)	40±2·3	−4±2·5*
		Sodium cyanide (mM)	28±1·7 (6)	20±2·2 (6)	11±2·3	2±2·7*
		Sodium salicylate (5 mM)	42±0·9 (6)	16±0·7 (6)	25±1·1	−2±1·7*

* $P > 0·05$. † $P < 0·05 > 0·01$.

unlike sodium salicylate (*o*-hydroxybenzoate), did not stimulate glucose uptake (Table 9).

In a medium buffered with phosphate (initial pH 7·4) the uptake of glucose by isolated diaphragm is not increased by 2:4-dinitrophenol, sodium salicylate or sodium cyanide (Table 9), results which are in general agreement with those of Villee *et al.* (1949) and Pierce & Field (1949) for 2:4-dinitrophenol and of Smith & Jeffrey (1956) for sodium salicylate.

Uptake of glucose in the presence of insulin

As was shown by Gemmill & Hamman (1941), the uptake of glucose by isolated diaphragm is very greatly increased by insulin *in vitro* (Table 10). The maximum effect of insulin on glucose uptake is seen with the concentration used (0·1 unit/ml.) and it is much greater than that produced either by anaerobiosis or by cell poisons at the concentrations used. When diaphragm is incubated with insulin (0·1 unit/ml.) under anaerobic conditions the level of glucose uptake does not differ significantly from that attained when insulin is present under aerobic conditions (Table 10). Furthermore, insulin still stimulates glucose uptake in the presence of sodium arsenite, sodium salicylate or 2:4-dinitrophenol, though under these conditions the uptake is less than that elicited by insulin alone (Table 11). When insulin and cyanide are present together the uptake of glucose is greater than that produced by insulin or cyanide separately. Insulin at a much lower concentration (10^{-3} unit/ml.) has no significant effect on glucose uptake under anaerobic conditions although it is still able to stimulate glucose uptake under aerobic conditions (Table 10).

Potassium loss from isolated diaphgram

When isolated diaphragm is incubated under aerobic conditions in media buffered with either bicarbonate or phosphate a small but significant amount of potassium is lost from the muscle (Table 12). This loss of potassium takes place during the initial 20 min. of incubation; thereafter no further loss occurs (Fig. 2). Kamminga, Willebrands, Groen & Blickman (1950) have claimed that hemidiaphragms incubated aerobically in bicarbonate medium take up some potassium from the medium and also that insulin enhances this transfer. Despite the fact that our experiments were carried out under conditions apparently very similar to those used by Kamminga *et al.* a loss of potassium from the tissue always occurred, although this loss was perhaps smaller in the presence of insulin (Table 12).

Much more potassium is lost if diaphragm is incubated under anaerobic conditions (Table 12): the loss is apparently linear with time (Fig. 2). The marked loss of potassium induced by exclusion of oxygen confirms the results of Creese (1954) and Calkins *et al.* (1954) and is in agreement with the known effects of anaerobiosis on other tissues. Diaphragm incubated aerobically but in the presence of 2:4-dinitrophenol (50 μM or 0·25 mM), sodium arsenite (mM), sodium arsenate (10 mM), sodium azide (10 mM), sodium cyanide (mM) or sodium salicylate (5 mM) also loses considerably more potassium. The influence of these agents on potassium release, unlike their effect on glucose uptake, is evident whether phosphate medium or bicarbonate medium is used for the incubation (Table 12). Sodium *p*-hydroxybenzoate (5 mM), unlike sodium salicylate, does not promote loss of potassium. There does not appear to be any relation between the changes in glucose uptake and potassium loss which these agents effect (Table 12).

DISCUSSION

The present investigations have shown that oxygen lack, 2:4-dinitrophenol, sodium arsenite, sodium arsenate, sodium cyanide and sodium salicylate increase the uptake of glucose and loss of potassium by isolated rat diaphragm incubated in a medium buffered with bicarbonate. Although these factors may differ in many respects in their effects on metabolism they nevertheless have in common the property of inhibiting oxidative phosphorylation. It seems reasonable to relate their effects on glucose uptake and potassium loss to this property. The greatly increased loss of potassium from diaphragm under conditions where oxidative phosphorylation

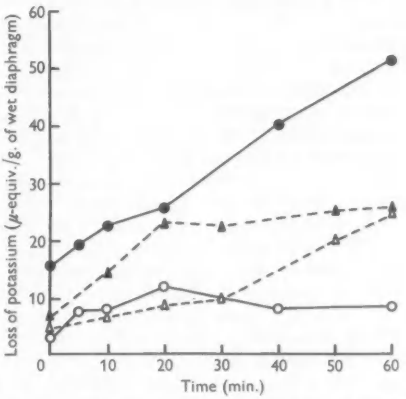

Fig. 2. Effect of anaerobiosis on loss of potassium from diaphragm. Incubations were made in bicarbonate medium under the following conditions: ●, anaerobic for 60 min.; ○, aerobic for 60 min.; ▲, anaerobic for 20 min. then aerobic for 40 min.; △, aerobic for 20 min. then anaerobic for 40 min.

is impaired is in keeping with the view that the maintenance of a high intracellular-potassium concentration is dependent upon the selective extrusion of sodium by an active process (see for example, Hodgkin, 1958). The lack of correlation between levels of glucose uptake and potassium release under anaerobic conditions or where cell poisons are present makes it unlikely that changes in glucose uptake occur as a consequence of loss of potassium.

The uptake of glucose by very many cells and tissue preparations *in vitro* is known to be greater under anaerobic conditions, as was first recognized by Pasteur. Previous investigations had suggested that isolated diaphragm was exceptional in that it did not appear to exhibit an increased uptake of glucose under anaerobic conditions. Failure to demonstrate this manifestation of the Pasteur effect in diaphragm has now been shown to be due to the use of media buffered with phosphate. The stimulating effect of 2:4-dinitrophenol on glucose uptake is also lacking in a phosphate-buffered medium. This difference in the response of isolated diaphragm to anaerobiosis or cell poisons such as 2:4-dinitrophenol in media buffered with phosphate or bicarbonate appears to be related to the poor buffering power of phosphate medium. The unsuitability of phosphate buffers for the incubation of tissues which exhibit a high rate of glycolysis was emphasized by Dixon (1934); it is also evident, for example, in the experiments of Stadie & Zapp (1947) and of Light & Simpson (1956).

A number of factors may have to be taken into account in explanation of the increased rates of glucose utilization and glycolysis by tissues under anaerobic conditions. These include: depression of glycolysis under aerobic conditions by lack of inorganic phosphate (Johnson, 1941; Lynen, 1941) or of adenosine diphosphate (Krebs, 1954); inactivation of phosphohexokinase under aerobic conditions (Engelhardt & Sakov, 1943); shortage of coenzyme I for aerobic glycolysis (Reiner, 1947); and the lack of availability of mitochondrial adenosine triphosphate for phosphorylation of glucose under aerobic conditions (Wu & Racker, 1957). Which of these factors is of importance to the increased uptake of glucose by diaphragm (or, indeed, by other tissues) under anaerobic conditions is not known. Since inorganic phosphate can stimulate the uptake of glucose by diaphragm under aerobic conditions, availability of inorganic phosphate might appear to be an important factor. However, inorganic phosphate has been shown to inhibit oxidative phosphorylation by rat-liver mitochondria (Hunter & Ford, 1955) and its effect on glucose uptake by diaphragm under aerobic conditions may be related to this action rather than to its participation in glycolysis.

The factors listed above can account for the enhanced utilization of glucose under anaerobic conditions only if the access of glucose to hexokinase is not impeded by any kind of permeability barrier. This may be so for some cells, such as the ascites-tumour cell, which shows augmented glucose uptake under anaerobic conditions (Wu & Racker, 1957) and in which glucose utilization is apparently not limited by the rate of entry of glucose into the cell (Crane, Field & Cori, 1957). Park *et al.* (1955) believe that glucose utilization in the isolated diaphragm, however, is limited by the rate of entry of glucose into the muscle cell. Thus the factors listed above may not account for the augmented uptake of glucose by isolated diaphragm under anaerobic conditions. The factors which regulate glucose uptake in muscle, which responds to insulin with increased uptake, obviously differ in some respect from those involved in cells such as the ascites-tumour cell which do not respond to insulin. Thus the mechanism of the Pasteur effect in a tissue such as muscle in which glucose utilization is affected by insulin may differ from that of a tissue or cell which is not affected by insulin.

Levine & Goldstein (1955) and Park *et al.* (1955) believe that insulin increases the uptake of glucose by muscle (including isolated diaphragm) by accelerating the entry of glucose into the cell. In an accompanying paper (Randle & Smith, 1958) we present evidence which suggests that anaerobiosis and substances which inhibit oxidative phosphorylation also accelerate the entry of glucose into the cells of isolated diaphragm. We have therefore concluded that the entry of glucose into the cells of this preparation is restrained under basal conditions by a process dependent upon a supply of energy-rich phosphate. On the other hand, it is well known that insulin does not inhibit oxidative phosphorylation (Stadie, 1954). The mechanism of its accelerating effect on the entry of glucose into the muscle cell may therefore differ from that of anaerobiosis and that of substances which do inhibit oxidative phosphorylation. One possibility is that insulin restricts the access of energy-rich phosphate to the process concerned with the regulation of glucose entry (Randle & Smith, 1957).

The entry of glucose into the cells of isolated diaphragm appears to be accelerated by anaerobiosis and cell poisons in both bicarbonate and phosphate media (Randle & Smith, 1958). This could account for the augmented glucose utilization which these factors promote in bicarbonate medium, but it fails to explain why glucose utilization is not increased in phosphate medium. This discrepancy could be explained if the intracellular utilization of glucose was rate-limiting in phosphate medium, but this is unlikely because free

glucose does not accumulate in the muscle under these conditions (Randle & Smith, 1958). Another explanation which may prove more fruitful is that some utilization of glucose may occur at the surface of the cells of isolated diaphragm (i.e. without penetration of the cell by glucose). Thus accelerated entry of glucose would not be associated with an increased rate of glucose utilization if at the same time there was a proportionate reduction in the rate of utilization of glucose at the cell surface. Although it is thought that the enzymes responsible for glycolysis in muscle occur in the sarcoplasm (see, for example, Bailey, 1954; Perry, 1956), Shaw & Stadie (1957) have recently suggested that the formation of lactic acid from glucose by isolated diaphragm does not depend on penetration of the cell by glucose but may occur at the cell surface. If this is so then the accelerated entry of glucose which anaerobiosis and cell poisons effect may be incidental and of little significance in relation to the augmented glucose utilization induced by these factors in bicarbonate medium. Clearly, further information about the localization in the muscle cell of processes concerned with glucose utilization is needed before the mechanism of the Pasteur effect in diaphragm can be elucidated.

At a concentration of 0·1 unit/ml., insulin can still enhance the uptake of glucose by isolated diaphragm in a medium buffered with bicarbonate when incubated under anaerobic conditions or in the presence of substances which inhibit oxidative phosphorylation. Ottaway (1955) has claimed that insulin (0·2 unit/ml.) fails to stimulate glucose uptake when diaphragm is incubated in a bicarbonate medium under anaerobic conditions. This apparent discrepancy between our results and those of Ottaway may result from the much smaller increase in glucose uptake which insulin seems to effect under aerobic conditions in Ottaway's experiments (Ottaway, 1951). This explanation is supported by our finding that insulin at a much lower concentration (10^{-3} unit/ml.), which under aerobic conditions induces a much smaller increase in glucose uptake, no longer stimulates glucose uptake under anaerobic conditions.

The present investigations have been concerned solely with the influence of certain factors on glucose utilization by muscle *in vitro*. Some indication that they may have a similar effect *in vivo* is provided by the observations that sodium salicylate (*o*-hydroxybenzoate) or acetylsalicylic acid raise the metabolic rate in normal people (Cochran, 1952) and diabetic patients (Reid, Macdougall & Andrews, 1957), and lower the blood-sugar level and reduce glycosuria in diabetic patients (Reid *et al.* 1957) and in several forms of experimental diabetes (Ingle, 1950; Smith, 1952; Smith, Meade & Bornstein, 1952); sodium *p*-hydroxybenzoate is without effect on the metabolic rate in human beings (Cochran, 1954). The present observation that salicylate, but not *p*-hydroxybenzoate, is capable of stimulating the uptake of glucose by diaphragm has led us to suggest elsewhere that these effects of salicylate on metabolic rate, blood-sugar level and glycosuria may result from an increased utilization of glucose by muscle (Manchester, Randle & Smith, 1958).

SUMMARY

1. Anaerobiosis stimulates the uptake of glucose by isolated rat diaphragm incubated in a medium buffered with bicarbonate. This effect is not observed in media buffered with phosphate or tris (of initial pH 7·4). The lack of response to anaerobiosis in the latter media appears to be related to the marked fall in pH which occurs during incubation.

2. Under aerobic conditions uptake of glucose by diaphragm in bicarbonate medium is increased by 2:4-dinitrophenol (50 μM or 0·25 mM), sodium arsenite (mM), sodium arsenate (10 mM), sodium cyanide (mM) and sodium salicylate (5 mM). Sodium azide (mM or 10 mM) and sodium *p*-hydroxybenzoate (5 mM) do not augment glucose uptake. In a medium buffered with phosphate, 2:4-dinitrophenol (0·25 mM), sodium salicylate (5 mM) and sodium cyanide (mM) fail to stimulate glucose uptake.

3. In bicarbonate medium the uptake of glucose by diaphragm in the presence of 2:4-dinitrophenol, sodium arsenite or sodium salicylate is increased still further by the addition of insulin (0·1 unit/ml.), though the uptake of glucose achieved under these conditions is less than that with insulin alone. The levels of uptake with insulin under either aerobic or anaerobic conditions were of the same order. In the presence of both insulin and cyanide the uptake of glucose is greater than when either is present by itself.

4. There is a small loss of potassium from diaphragm incubated under aerobic conditions either in bicarbonate or in phosphate medium. The loss of potassium in either medium is very much greater under anaerobic conditions or in the presence of 2:4-dinitrophenol, sodium arsenite, sodium arsenate, sodium cyanide, sodium salicylate or sodium azide at the above concentrations. Insulin effected a slight reduction in the loss of potassium under aerobic conditions. There was no obvious quantitative relation between the loss of potassium from the muscle and the change in glucose uptake.

5. The significance of these results in relation to the mode of uptake of glucose by diaphragm, to the

Pasteur effect in diaphragm and to the mechanism of action of insulin on diaphragm is discussed.

We wish to thank Professor F. G. Young, F.R.S., for much helpful discussion and encouragement. We are indebted to Dr G. D. Greville for valuable discussions and for a sample of highly purified 2:4-dinitrophenol. Miss G. M. Bixley is thanked for skilled technical assistance and Miss M. Thomas for carrying out hypophysectomies. We are grateful to Boots Pure Drug Co. Ltd. and to Dr K. Hallas Møller of the Novo Terapeutisk Laboratories, Copenhagen, for generous gifts of crystalline insulin. The cost of this work was largely defrayed by a grant for research expenses from the Medical Research Council to one of us (P.J.R.).

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Regulation of Glucose Uptake by Muscle

2. THE EFFECTS OF INSULIN, ANAEROBIOSIS AND CELL POISONS ON THE PENETRATION OF ISOLATED RAT DIAPHRAGM BY SUGARS*

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The utilization of glucose by muscle is thought to be limited by the rate at which glucose enters the muscle cell and insulin is believed to stimulate glucose uptake by speeding its entry (Levine & Goldstein, 1955; Park, Bornstein & Post, 1955; Park & Johnson, 1955). We have shown that the uptake of glucose by isolated rat diaphragm incubated in a bicarbonate-buffered medium is increased by anoxia and by substances which inhibit oxidative phosphorylation, as well as by insulin (Randle & Smith, 1957, 1958). These observations led us to suggest that the entry of sugars such as glucose into the muscle cell is restrained under basal conditions by a process dependent upon a supply of energy-rich phosphate. We have now attempted to obtain more direct evidence in support of this suggestion by studying the effects of insulin, anaerobiosis and substances which inhibit oxidative phosphorylation on the accumulation of glucose and xylose in isolated diaphragm.

We will express the amount of glucose or xylose accumulating in diaphragm as a space, the glucose or xylose space being that fraction of the volume of the tissue which appears to contain fluid of the same specific gravity and glucose or xylose content as the incubation medium. Interpretation of these results in terms of the distribution of sugar between extracellular and intracellular water involves comparing the glucose or xylose space with the volume of extracellular fluid held by the tissue. Glucose or xylose are deemed to be present in intracellular water only if the glucose or xylose space exceeds the volume of extracellular fluid. The volume of extracellular fluid may be estimated by measuring the space occupied by substances which are believed not to enter the cells under normal conditions. We have used both inulin and the thiosulphate ion for this purpose, though we have found, as others have (Kipnis & Cori, 1957), that thiosulphate yields higher values for the volume of extracellular fluid than does inulin. The reliance that can be placed on either the thiosulphate space or the inulin space alone is therefore uncertain and the conclusions which we draw are, for the most part, based on both estimates of the volume of extracellular fluid. In most of the experiments we have used a diaphragm preparation, first described by Kipnis & Cori (1957), in which no muscle fibres are cut. The advantage of this preparation is that its spaces are apparently similar to those of the muscle *in vivo* (Kipnis & Cori, 1957).

* Part 1: Randle & Smith, 1958.

METHODS AND PROCEDURE

Incubation media. The composition of the basal media is given in Table 1. A.R.-grade chemicals were used if commercially available. Inulin and D-xylose were obtained from T. Kerfoot and Co. Ltd.

Table 1. *Composition of basal media used for incubation of isolated diaphragm*

Media containing bicarbonate were gassed with O_2+CO_2 or N_2+CO_2 (95:5); pH 7·4 at 37°. Phosphate medium was gassed with O_2 or N_2; pH 7·4 at 37°. When glucose or thiosulphate space was measured D-glucose was added (2·5 mg./ml.); for xylose space, D-xylose (1·5 or 5 mg./ml.) and D-glucose (1 mg./ml.) were added; inulin space was measured in the presence of inulin (2 or 4 mg./ml.) and D-glucose (1 or 2·5 mg./ml.). The units m-osmoles have been suggested and defined by Gamble (1950).

Medium	Composition (m-equiv./l.)									Calc. osmolarity† (m-osmoles/l.)
	Na	K	Ca	Mg	Cl	HCO_3	PO_4*	SO_4	S_2O_3	
Bicarbonate‡	124·8	5·2	2·7	2·6	105·7	27·2	1·8	0·6	—	281
Bicarbonate–thiosulphate I	134·8	5·2	2·7	2·6	75·7	27·2	1·8	0·6	40	281
Bicarbonate–thiosulphate II	151·5	5·2	2·7	2·6	92·4	27·2	1·8	0·6	40	314
Phosphate	143·2	5·1	2·7	2·6	132·8	—	18·2	0·6	—	309
Phosphate–thiosulphate	169·9	5·1	2·7	2·6	119·5	—	18·2	0·6	40	342
High phosphate–bicarbonate	133·0	5·2	2·7	2·6	77·9	27·2	37·8	0·6	—	281

* Assuming valency of 1·8 at pH 7·4.
† Calculated for media containing 2·5 mg. of glucose/ml. or its equivalent.
‡ Gey & Gey (1936).

Cut-diaphragm preparation. Hemidiaphragms were prepared and incubated as described in the preceding paper (Randle & Smith, 1958). After incubation each hemidiaphragm was removed from the flask, and lightly blotted, and weighed on a torsion balance and frozen immediately on an aluminium planchet resting on solid carbon dioxide.

Intact-diaphragm preparation. Animals were killed by decapitation and exsanguinated. The skin and fascia were dissected from the thoraco-abdominal wall, and the ribs and abdominal wall on either side of the diaphragm were divided, and the viscera separated from the diaphragm and the vertebral column transected above and below the diaphragm. The diaphragm, with its attachments to spine, ribs, xiphisternum and costal cartilages intact, was then washed in the appropriate incubation medium with continuous gassing. Each diaphragm was then slid into a 50 ml. conical flask containing 10 or 20 ml. of medium. The flasks were gassed with the appropriate gas or gas mixture, sealed with rubber stoppers and incubated with shaking in a water bath at 37°. Incubation was for 1 hr. unless otherwise stated. The tissue was then removed from the flask, the diaphragm (excepting the posterior portion) excised from the rib cage and blotted, and weighed and frozen as described for the cut preparation. If more than one analysis was made on the same diaphragm, the muscle was divided along the central tendon and the two hemidiaphragms were weighed and frozen separately.

Analytical methods

Glucose, xylose or inulin. Analyses of muscle for these substances were made on extracts prepared by grinding the frozen muscle rapidly to a paste with sand in a mortar, and adding 3 ml. of water and deproteinizing with $Zn(OH)_2$ (Somogyi, 1945). Samples of medium after incubation were suitably diluted and deproteinized in the same way. Glucose was determined photometrically with glucose oxidase, peroxidase and *o*-dianisidine (see Randle & Smith, 1958), xylose photometrically by the *p*-bromoaniline method (Roe & Rice, 1948) and inulin photometrically after hydrolysis and reaction with resorcinol (Roe, Epstein & Goldstein, 1949).

Total water and thiosulphate. These were determined in the same sample of muscle. The sample was weighed wet, and freeze-dried and reweighed. The dried tissue was then ground to a powder with sand in a mortar and deproteinized by addition of 5 ml. of tungstic acid (Van Slyke & Hawkins, 1928). Samples of medium after incubation were similarly deproteinized. Thiosulphate in the extract was then assayed iodometrically (Newman, Gilman & Philips, 1946):

$$\text{Space (ml./100 g.)} = \frac{\text{muscle content (g./100 g. of wet muscle)}}{\text{medium concentration (g./ml.)}}.$$

$$\text{Total water (ml./100 g.)} = \frac{\text{wet wt.} - \text{dry wt.}}{\text{wet wt.}} \times 100.$$

Other experimental details and the statistical methods have been given in the preceding paper (Randle & Smith, 1958).

RESULTS

Parameters of isolated diaphragm

(1) *Total water content.* The intact preparation always contained 74 ml. of water/100 g. of wet tissue, whether incubated in bicarbonate medium or bicarbonate–thiosulphate medium I or II (Table 2) or in phosphate–thiosulphate medium (Table 4). The cut preparation incubated in bicarbonate–thiosulphate medium II also contained 74 ml. of water/100 g. The total water content of the cut preparation incubated in bicarbonate medium was not determined in these experiments, though the same value of 74 ml./100 g. had previously been found by drying at 110° (Randle, 1956). Values of 77–78 ml./100 g. were obtained by Creese (1954) and Kipnis & Cori (1957) by drying at 100° or 105°.

(2) *Extracellular volume (thiosulphate and inulin spaces).* The thiosulphate space of intact diaphragm was 21–25 ml./100 g. (Tables 2 and 3). The inulin

Table 2. *Parameters of isolated diaphragm*

For composition of media see Table 1. The number of observations is given in parentheses.

Medium	Parameter	Space. Mean ± S.E.M. (ml./100 g. of wet diaphragm)	
		Cut diaphragm	Intact diaphragm
Bicarbonate	Total water	(74*)	74 ± 0·7 (6)
	Inulin space	30 ± 1·1 (5)	12 ± 0·7 (11)
	Glucose space	—	13 ± 0·7 (5)
	Xylose space	52 ± 0·7 (3)	25 ± 0·3 (5)
Bicarbonate–thiosulphate I	Total water	—	74 ± 0·6 (6)
	Inulin space	—	12 ± 0·7 (5)
	Xylose space	—	28 ± 1·0 (6)
Bicarbonate–thiosulphate II	Total water	74 ± 0·6 (12)	74 ± 0·2 (51)
	Thiosulphate space	37 ± 0·9 (12)	23 ± 0·3 (39)
	Inulin space	—	14 ± 0·5 (6)
	Glucose space	29 ± 0·8 (12)	14 ± 0·2 (5)
	Xylose space	—	34 ± 0·7 (6)
High phosphate–bicarbonate	Xylose space	—	32 ± 1·4 (5)

* Determined by drying at 110° by Randle (1956).

Table 3. *Influence of insulin, anaerobiosis or cell poisons on the parameters of intact diaphragm incubated in bicarbonate-buffered medium*

Diaphragms were incubated in bicarbonate–thiosulphate medium II (for composition see Table 1). Glucose uptake, total water and thiosulphate and glucose spaces were measured in the same experiment; inulin spaces were measured separately. The number of observations is given in parentheses.

Expt. no.	Addition	Space. Mean ± S.E.M. (ml. of water/100 g. of wet diaphragm)				Glucose uptake. Mean ± S.E.M. (mg. of glucose/hr. of incubation)
		Total water	Thiosulphate	Glucose	Inulin	
1	None (aerobic control)	74 ± 0·92 (5)	21 ± 1·05 (5)	16 ± 0·97 (5)	14 ± 0·48 (6)	4·4 ± 0·37 (5)
	Insulin (0·1 unit/ml.)	75 ± 0·53 (5)	17 ± 1·18 (5)	14 ± 1·41 (5)	12 ± 0·0 (3)	6·2 ± 0·50 (5)
	Significance of difference (*P*)	< 0·4 > 0·3	< 0·05 > 0·02	< 0·3 > 0·2	< 0·01 > 0·001	< 0·02 > 0·01
2	None (aerobic control)	74 ± 0·30 (18)	25 ± 0·47 (18)	17 ± 0·73 (18)	14 ± 0·48 (6)	2·5 ± 0·21 (18)
	None (anaerobic)	76 ± 0·25 (18)	22 ± 0·81 (18)	11 ± 0·45 (18)	10 ± 0·42 (3)	3·1 ± 0·14 (18)
	Significance of difference (*P*)	< 0·001	< 0·01 > 0·001	< 0·001	< 0·001	< 0·05 > 0·01
3	None (aerobic control)	73 ± 0·31 (5)	21 ± 0·71 (5)	15 ± 0·84 (5)	14 ± 0·48 (6)	2·6 ± 0·14 (5)
	2:4-Dinitrophenol (0·25 mM)	77 ± 0·71 (5)	24 ± 1·20 (5)	30 ± 0·50 (5)	13 ± 1·21 (3)	3·2 ± 0·15 (5)
	Significance of difference (*P*)	< 0·001	< 0·1 > 0·05	< 0·001	< 0·5 > 0·4	< 0·02 > 0·01
4	None (aerobic control)	73 ± 0·32 (6)	24 ± 0·61 (6)	14 ± 0·88 (6)	— —	2·9 ± 0·29 (6)
	2:4-Dinitrophenol (0·25 mM) + sodium fluoride (10 mM)	76 ± 0·36 (6)	27 ± 1·81 (6)	53 ± 2·90 (6)		2·0 ± 0·16 (6)
	Significance of difference (*P*)	< 0·001	< 0·2 > 0·1	< 0·001	—	< 0·05 > 0·02
5	None (aerobic control)	75 ± 0·59 (5)	21 ± 1·38 (5)	15 ± 1·05 (5)	14 ± 0·48 (6)	2·3 ± 0·64 (5)
	Sodium arsenite (mM)	78 ± 0·32 (5)	34 ± 2·14 (5)	36 ± 2·04 (5)	19 ± 0·71 (3)	5·5 ± 0·60 (5)
	Significance of difference (*P*)	< 0·01 > 0·001	< 0·001	< 0·001	< 0·001	< 0·01 > 0·001
6	None (aerobic control)	73 ± 1·00 (6)	23 ± 0·92 (6)	19 ± 1·79 (6)	14 ± 0·48 (6)	1·7 ± 0·35 (6)
	Sodium cyanide (mM)	75 ± 0·17 (6)	28 ± 1·39 (6)	19 ± 0·91 (6)	19 ± 1·21 (6)	3·8 ± 0·24 (6)
	Significance of difference (*P*)	< 0·1 > 0·05	< 0·02 > 0·01	—	< 0·01 > 0·001	< 0·001
7	None (aerobic control)	74 ± 0·48 (6)	22 ± 1·32 (6)	17 ± 2·01 (6)	14 ± 0·48 (6)	1·8 ± 0·29 (6)
	Sodium salicylate (5 mM)	75 ± 0·63 (6)	25 ± 1·30 (6)	18 ± 2·20 (6)	11 ± 0·39 (6)	3·8 ± 0·24 (6)
	Significance of difference (*P*)	< 0·3 > 0·2	< 0·2 > 0·1	< 0·8 > 0·7	< 0·01 > 0·001	< 0·001

Table 4. *Influence of insulin, anaerobiosis or cell poisons on the parameters of intact diaphragm incubated in phosphate-buffered medium*

Diaphragms were incubated in phosphate–thiosulphate medium (for composition see Table 1). The number of observations is given in parentheses.

Expt. no.	Addition	Space. Mean ± S.E.M. (ml. of water/100 g. of wet diaphragm)			Glucose uptake. Mean ± S.E.M. (mg. of glucose/hr. of incubation)
		Total water	Thiosulphate	Glucose	
1	None (aerobic control)	74 ± 0·63 (6)	25 ± 0·73 (6)	17 ± 1·11 (6)	2·8 ± 0·13 (6)
	None (anaerobic)	77 ± 0·18 (6)	25 ± 0·66 (6)	15 ± 0·52 (6)	3·4 ± 0·31 (6)
	Significance of difference (P)	<0·01>0·001	—	<0·2>0·1	<0·1>0·05
2	None (aerobic control)	73 ± 0·69 (6)	25 ± 0·81 (6)	18 ± 0·63 (6)	2·6 ± 0·13 (6)
	2:4-Dinitrophenol (0·25 mM)	76 ± 0·55 (6)	34 ± 1·11 (6)	37 ± 2·50 (6)	2·2 ± 0·16 (6)
	Significance of difference (P)	<0·01>0·001	<0·001	<0·001	<0·1>0·05
3	None (aerobic control)	—	—	27 ± 1·12 (6)	3·5 ± 0·22 (6)
	Sodium salicylate (5 mM)	—	—	29 ± 2·09 (6)	3·8 ± 0·41 (6)
	Significance of difference (P)	—	—	<0·5>0·4	<0·6>0·5

space was 12–14 ml./100 g. whether determined in the presence or in the absence of thiosulphate (Tables 2 and 3). The thiosulphate and inulin spaces of the cut preparation were both very much higher (37 and 30 ml./100 g. respectively), though a similar discrepancy was apparent as Kipnis & Cori (1957) also found. It seems unlikely that this disparity between the two estimates of extracellular water is due to incomplete equilibration of the tissue with inulin during the period of incubation (1 hr.) as Creese has shown that the inulin space attains a maximum value within this time. Thiosulphate must therefore be in equilibrium with a larger fraction of the tissue than inulin.

(3) *Glucose and xylose spaces.* The glucose spaces of the intact and cut preparations incubated in bicarbonate-buffered media (13–14 and 28 ml./100 g. respectively; Tables 2 and 3) were very similar to the corresponding inulin spaces though considerably smaller than the corresponding thiosulphate spaces; but by either estimate of the volume of extracellular fluid there was apparently no free glucose in intracellular water. Essentially similar results were obtained with the intact preparation in phosphate-buffered medium (Table 4). On the other hand, the xylose space of either intact or cut diaphragm in bicarbonate medium (26 and 52 ml./100 g. respectively; Table 2) exceeded both estimates of the volume of extracellular fluid. Some xylose therefore entered intracellular water under basal conditions. Kipnis & Cori (1957) obtained higher figures for the xylose spaces of both intact and cut preparations but they used medium containing thiosulphate and a high concentration of phosphate. Phosphate in high concentration (20 mM) increases the xylose space of intact diaphragm and so to a smaller extent does thiosulphate (20 mM) (Table 2). The accumulation of xylose in either diaphragm preparation was found to be rapid, being 80–85% complete after incubation for 5–10 min. in bicarbonate medium (Figs. 1–3). For a reason which is not clear, penetration of the intact diaphragm by xylose was considerably faster, though ultimately less extensive, than in the experiments of Kipnis & Cori (1957).

(4) *Other effects of thiosulphate.* In view of the effect of thiosulphate on the xylose space just noted and because it also increases the uptake of glucose by cut diaphragm (Randle & Smith, unpublished observations) it was important to show whether thiosulphate affected any other parameters of diaphragm. The comparisons given in Table 2 indicate that thiosulphate does not influence the inulin or glucose spaces or the total water of intact diaphragm incubated in bicarbonate-buffered medium. In separate experiments it was also shown that thiosulphate did not appreciably modify the influence of 2:4-dinitrophenol, sodium cyanide or sodium salicylate on the glucose space of the intact diaphragm incubated in bicarbonate-buffered medium.

Effects of insulin, anaerobiosis and cell poisons on parameters of intact diaphragm

In studying the effects of insulin, anaerobiosis or cell poisons, the glucose space, thiosulphate space and total water were measured in the same experiment. The inulin space was determined in separate experiments but in the presence of thiosulphate to validate comparison with the thiosulphate and glucose spaces. When the xylose space was measured thiosulphate was excluded from the medium because of its effect on the xylose space and inulin was also omitted because it interferes with the estimation of xylose.

(1) *Glucose space in bicarbonate-buffered medium.* The results in Table 3 show that insulin (0·1 unit/ml.) or anaerobiosis reduced the glucose space, but that this change was associated with a similar fall

in the volume of extracellular fluid as measured with both inulin and thiosulphate. Neither sodium cyanide (mM) nor sodium salicylate (5 mM) affected the glucose space, though small changes in the extracellular volume occurred when these substances were present. Hence it may be concluded that none of these agents caused free glucose to accumulate in intracellular water. On the other hand 2:4-dinitrophenol (0·25 mM) increased the glucose space to a value exceeding both the inulin and the thiosulphate spaces and with both 2:4-dinitrophenol (0·25 mM) and sodium fluoride (10 mM) the glucose space increased still further. Free glucose was therefore present in the intracellular water under these conditions. The effect of sodium arsenite (mM) was anomalous in that although it more than doubled the glucose space the thiosulphate space also rose to a comparable value. The inulin space was much smaller than the glucose space or the thiosulphate space in the presence of arsenite. If the inulin space is to be regarded as a more reliable estimate of the volume of extracellular fluid then free glucose accumulated in intracellular water in the presence of arsenite.

(2) *Glucose uptake in bicarbonate-buffered medium.* The uptake of glucose from the medium by intact diaphragm was increased by insulin, anaerobiosis, 2:4-dinitrophenol, sodium arsenite, sodium cyanide and sodium salicylate (Table 3). These factors thus had effects on the uptake of glucose by the intact diaphragm which were similar to those obtained with the cut preparation (Randle & Smith, 1958). It should, of course, be borne in mind that with the intact preparation structures other than the diaphragm itself presumably take up glucose from the medium. The absolute values of the glucose uptakes with the two preparations may not therefore be strictly comparable. If sodium fluoride was

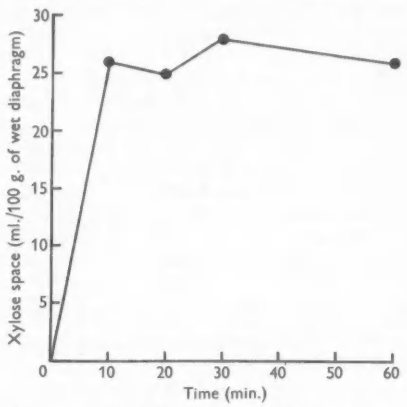

Fig. 1. Accumulation of xylose in intact diaphragm. Incubations were made in bicarbonate medium (for composition see Table 1) to which was added D-xylose (5 mg./ml.) and glucose (1 mg./ml.).

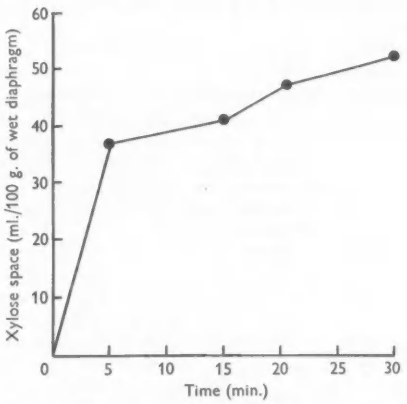

Fig. 2. Accumulation of xylose in cut diaphragm. Incubations were made in bicarbonate medium (for composition see Table 1) to which was added D-xylose (5 mg./ml.) and glucose (1 mg./ml.).

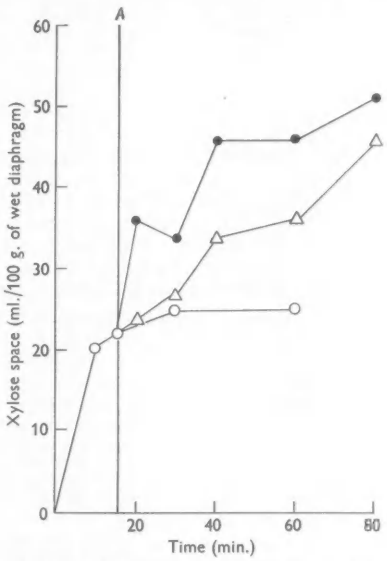

Fig. 3. Effect of insulin and salicylate on accumulation of xylose in intact diaphragm. Incubations were made in bicarbonate medium (for composition see Table 1) to which was added D-xylose (5 mg./ml.) and glucose (1 mg./ml.). ○, Aerobic control; △, salicylate added at *A* (to 5 mM); ●, insulin added at *A* (to 0·1 unit/ml.).

present in addition to the 2:4-dinitrophenol the glucose uptake was lower than in the control. Similar experiments with the cut diaphragm showed that the actual utilization of glucose (as distinct from the disappearance of glucose from the medium) in the presence of 2:4-dinitrophenol plus sodium fluoride was very much lower than that in the control. Much of the glucose which disappeared from the medium under these conditions accumulated in the muscle (Table 5).

(3) *Glucose space and glucose uptake in phosphate-buffered medium.* The effects of anaerobiosis, 2:4-dinitrophenol or sodium salicylate on the glucose space of intact diaphragm incubated in phosphate-buffered medium (Table 4) did not differ appreciably from their effects in bicarbonate-buffered medium (Table 3). On the other hand, dinitrophenol and salicylate failed to increase glucose uptake by intact diaphragm in phosphate-buffered medium (Table 4), in agreement with the results obtained previously with cut diaphragm (Randle & Smith, 1958).

(4) *Xylose space in bicarbonate or phosphate-buffered media.* The xylose space of the intact diaphragm preparation was expanded by insulin, 2:4-dinitrophenol, sodium arsenite, sodium cyanide or sodium salicylate or by exclusion of oxygen (Table 6). Of these factors arsenite and cyanide increased the xylose space to the greatest extent. They were also the only factors to increase the inulin and thiosulphate spaces (Table 3); the two effects may be associated. Moreover, insulin, 2:4-dinitrophenol and sodium salicylate increased the xylose space in phosphate medium (Table 6). Thus in every instance insulin, anaerobiosis and cell poisons expanded the xylose space to a value greatly in excess of the volume of extracellular fluid as estimated with either inulin or thiosulphate. It must therefore be concluded that each of these agents promotes the entry of xylose into intracellular water.

The rate of xylose penetration under the influence of insulin or salicylate was studied also. In this experiment intact diaphragms were first incubated for 10 min. in bicarbonate medium without insulin or salicylate to allow basal penetration to occur. Insulin or salicylate was then added and further estimations of xylose space were made at intervals over a total period of 1 hr. Fig. 3 shows that additional xylose immediately entered the

Table 5. *Effect of 2:4-dinitrophenol plus sodium fluoride on the parameters of cut diaphragm*

Hemidiaphragms were incubated in bicarbonate–thiosulphate medium II (for composition see Table 1). Glucose utilization was calculated by correcting the glucose uptake for free glucose present in the muscle.

	Space. Mean ± S.E.M. (ml./100 g. of wet diaphragm)			Glucose uptake and utilization. Mean ± S.E.M. (mg. of glucose/g. of wet diaphragm/hr. of incubation)		
Addition to medium	Total water	Thiosulphate	Glucose	Uptake	Utilization	No. of observations
None (aerobic control)	74 ± 0·52	32 ± 0·91	27 ± 1·41	3·7 ± 0·32	3·7 ± 0·32	6
2:4-Dinitrophenol (0·25 mM) + sodium fluoride (10 mM)	78 ± 0·41	45 ± 8·09	61 ± 0·95	1·7 ± 0·28	0·9 ± 0·28	6
Significance of difference (P)	<0·001	<0·3>0·2	<0·001	<0·001	<0·001	

Table 6. *Influence of insulin, anaerobiosis or cell poisons on the xylose space of intact diaphragm*

For composition of media see Table 1. The number of observations is given in parentheses.

Medium	Addition	Xylose space. Mean ± S.E.M. (ml. of water/100 g. of wet diaphragm)	*Difference (from control) ± S.E. of difference
Bicarbonate	None (aerobic control)	25 ± 0·4 (16)	—
	Insulin (0·1 unit/ml.)	48 ± 1 (4)	23 ± 1
	None (anaerobic)	44 ± 1 (3)	19 ± 1
	2:4-Dinitrophenol (0·25 mM)	48 ± 1 (3)	23 ± 1
	2:4-Dinitrophenol (0·25 mM) + sodium fluoride (10 mM)	44 ± 1 (4)	19 ± 1
	Sodium arsenite (mM)	66 ± 2 (3)	41 ± 2
	Sodium cyanide (mM)	52 ± 2 (4)	27 ± 2
	Sodium salicylate (5 mM)	44 ± 3 (3)	19 ± 3
Phosphate	None (aerobic control)	26 ± 2 (3)	—
	Insulin (0·1 unit/ml.)	46 ± 1 (3)	20 ± 3
	2:4-Dinitrophenol (0·25 mM)	49 ± 0·6 (3)	23 ± 2
	Sodium salicylate (5 mM)	50 ± 2 (3)	24 ± 3

* In every case the significance (P) of the difference is <0·001.

tissue in both cases, the rate of penetration being greater with insulin than with salicylate. The values ultimately attained after 1 hr. of incubation were, however, similar in the two cases.

DISCUSSION

We showed in the preceding paper that insulin, anaerobiosis and a number of cell poisons all enhance the uptake of glucose by isolated rat diaphragm (Randle & Smith, 1958). Levine & Goldstein (1955) and Park *et al.* (1955) believe that the uptake of glucose by muscle such as diaphragm occurs in two stages: (i) penetration of the muscle cell by glucose, and (ii) metabolism of glucose within the cell; and that it is the first of these which is normally rate-limiting. We therefore concluded that insulin, anaerobiosis and cell poisons such as 2:4-dinitrophenol or salicylate all act by stimulating the entry of glucose into the muscle cell, possibly by inhibiting a process which restrains the penetration of glucose under basal conditions. The results recorded here give further support for this view.

If intracellular utilization of glucose can proceed under basal conditions at a faster rate than entry, then little or no free glucose would be expected to accumulate within the cells. This was indeed shown to be the case with the cut-diaphragm preparation by Park *et al.* (1955) and the present investigation has demonstrated that the same is true for the intact diaphragm. Furthermore, the distribution of free glucose remained extracellular under anaerobic conditions or in the presence of insulin, cyanide or salicylate, even though these factors markedly enhanced glucose uptake. Hence either the potential rate of intracellular utilization of glucose must greatly exceed the rate of entry under these conditions or, alternatively, glucose does not enter the cell as such. Free glucose was detected within the cell, however, when diaphragm was incubated in the presence of 2:4-dinitrophenol, especially when sodium fluoride was also present. Thus unless the mechanism of entry of glucose in the presence of these substances is abnormal it seems likely that glucose does enter intracellular water as the free sugar, accumulation occurring only when utilization is inhibited to such an extent that it becomes rate-limiting. The results of measurements of glucose uptake and glucose space of intact diaphragm are therefore compatible with the view that insulin, anaerobiosis or substances which inhibit oxidative phosphorylation promote glucose uptake by accelerating the entry of glucose into the cell.

This view is strengthened by the results of experiments with xylose. D-Xylose does not appear to be utilized by isolated rat diaphragm (Kipnis & Cori, 1957). Consequently the xylose space may be used to measure the rate and extent of penetration of diaphragm by this sugar. Under basal conditions xylose rapidly enters a small volume (10–15%) of intracellular water; thereafter further penetration is very slow. This observation suggests that a barrier within the cell confines xylose to a small fraction of intracellular water under basal conditions (cf. Helmreich & Cori, 1957). Insulin, lack of oxygen and the cell poisons 2:4-dinitrophenol, arsenite, cyanide and salicylate all increase the fraction of intracellular water which xylose penetrates to 40–80%. Each of these agents except insulin has the property of inhibiting oxidative phosphorylation. It would therefore appear that the processes which confine xylose to a small fraction of intracellular water under basal conditions are dependent upon a supply of energy-rich phosphate compounds. Insulin, on the other hand, does not inhibit oxidative phosphorylation (Stadie, 1954), so the mechanism of its effect on glucose uptake and xylose entry presumably differs from that of factors which do inhibit oxidative phosphorylation. One possibility is that insulin may augment glucose or xylose penetration by restricting the access of energy-rich phosphate to a process concerned with the regulation of glucose or xylose entry (Randle & Smith, 1957, 1958). The observation that all of these factors increase both glucose uptake and entry of xylose in diaphragm incubated in a bicarbonate-buffered medium is compatible with the following views: (i) that glucose and xylose enter the muscle cell by similar processes, and (ii) that uptake of glucose in most instances is limited by the rate of its entry into the cell. Nevertheless the rates of glucose uptake elicited by these various factors are generally different in bicarbonate medium (Randle & Smith, 1958). If the views just outlined are correct then the rates of penetration of xylose with each of these factors should differ. This was shown to be the case for insulin and salicylate; insulin more markedly stimulated glucose uptake than salicylate (Randle & Smith, 1958) and also promoted a more rapid accumulation of xylose (Fig. 3). On the other hand, in phosphate medium 2:4-dinitrophenol and salicylate augment xylose accumulation without increasing glucose uptake. The failure of dinitrophenol and salicylate to increase glucose uptake in phosphate medium cannot result from a diminution in the utilization of glucose within the cell to such a level that it has become rate-limiting, for free glucose does not accumulate within the cell under these conditions. One must therefore conclude either that the processes which govern the entry of glucose and xylose into the muscle cell are different or else that glucose uptake does not necessarily reflect glucose entry, even when there

is no intracellular accumulation of glucose. One explanation of these anomalous findings in phosphate medium, which has been discussed more fully in the preceding paper (Randle & Smith, 1958), is provided by the suggestion of Shaw & Stadie (1957) that glucose may be utilized partly within the cell and partly at the cell surface. Thus increased entry of glucose into intracellular water would not be associated with increased glucose uptake if at the same time there was a more or less proportionate reduction in the rate of utilization of glucose at the surface of the cell.

Although the present investigations have provided further support for the view that it is the penetration of the muscle cell by glucose which is the rate-limiting step in the uptake of glucose by isolated diaphragm and that insulin or factors which inhibit oxidative phosphorylation augment glucose uptake by increasing penetration, there are, nevertheless, at least two unsatisfactory features in the evidence for this hypothesis. One is the assumption that the same processes govern the entry of glucose and non-utilizable sugars, such as xylose, into the muscle cell. The other is the assumption that glucose utilization in muscle is entirely intracellular. Clearly, further information about the process by which glucose enters the muscle cell and about the localization in muscle of the enzymes concerned with the metabolism of glucose is prerequisite for any explanation of the mechanism of action of insulin or of the Pasteur effect in this tissue.

SUMMARY

1. The accumulation of glucose or xylose in muscle has been studied *in vitro* with an isolated rat-diaphragm preparation in which none of the muscle fibres is cut.

2. The glucose space of this preparation does not exceed the volume of extracellular fluid when diaphragm is incubated aerobically in a bicarbonate-buffered medium. On the other hand, xylose appears to accumulate in about 10–15% of the intracellular water under these conditions.

3. Glucose remains extracellular in distribution when diaphragm is incubated anaerobically or in the presence of insulin (0·1 unit/ml.), sodium cyanide (mM) or sodium salicylate (5 mM), but accumulates within the cells in the presence of sodium arsenite (mM), 2:4-dinitrophenol (0·25 mM) or dinitrophenol (0·25 mM) plus fluoride (10 mM). Each of these factors causes a further accumulation of xylose within the muscle cell.

4. These results provide further support for the views that the entry of glucose into the muscle cell is normally the rate-limiting step in its metabolism; that the glucose entry is restrained by a process dependent on a supply of a substance generated during oxidative phosphorylation; and that insulin may promote glucose uptake by diaphragm by preventing access of this substance to the process regulating glucose entry.

We wish to thank Professor F. G. Young, F.R.S., for valuable discussion and encouragement. We are indebted to Dr G. D. Greville for a sample of highly purified 2:4-dinitrophenol. Miss G. M. Bixley is thanked for skilled technical assistance. We are grateful to Boots Pure Drug Co. Ltd. and to Dr K. Hallas Møller of the Novo Terapeutisk Laboratories, Copenhagen, for generous gifts of crystalline insulin. The cost of this work was largely defrayed by a grant for research expenses from the Medical Research Council to one of us (P.J.R.).

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Effect of Cyanide on the Biosynthesis of Ascorbic Acid by an Enzyme Preparation from Goat-Liver Tissue

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In exploring the mechanism of biosynthesis of ascorbic acid by the rat, King and his coworkers (Musulin, Tully, Longenecker & King, 1939; Longenecker, Musulin, Tully & King, 1939; Longenecker, Fricke & King, 1940) observed that rats treated with Chloretone (1:1:1-trichloro-2-methylpropan-2-ol) excreted more ascorbic acid than did the controls. By employing Chloretonized rats and isotopically labelled precursors they (Jackel, Mosbach, Burns & King, 1950; Horowitz, Doerschuk & King, 1952; Horowitz & King, 1953*a*, *b*) presented evidence for the following route of the synthesis:

D-Glucose → D-glucurono-γ-lactone → L-ascorbic acid.

Subsequently, Isherwood, Chen & Mapson (1954) and Mapson, Isherwood & Chen (1954) came to the conclusion from an extensive series of experiments that in plant and animal systems respectively the following sequences were involved:

(1) D-Galactose → D-galacturonic acid methyl ester → L-galactono-γ-lactone → L-ascorbic acid.

(2) D-Glucose → D-glucurono-γ-lactone → L-gulono-γ-lactone → L-ascorbic acid.

Hassan & Lehninger (1956) reported that rat-liver extracts containing the microsomes and soluble supernatant could convert D-glucurono-lactone, L-gulonolactone and also the corresponding free acids into L-ascorbic acid in the presence of cofactors.

In experiments previously carried out in this Laboratory it had been observed that rat-liver tissue incubated with sodium pyruvate showed a higher ascorbic acid value than did the controls incubated without the substrate (Roy, Roy & Guha, 1946). But in no experiment was the value so obtained higher than the original ascorbic acid content of the tissue before incubation, and it was possible therefore that pyruvic acid was not a direct precursor. In a search for a system in which a net synthesis over and above the original tissue ascorbic acid level takes place, Chatterjee, Ghosh, Ghosh & Guha (1957*a*) found, in later experiments, that a marked synthesis of ascorbic acid occurred in homogenates of rat-liver tissue, containing potassium cyanide, from D-glucurono-γ-lactone, the increase in ascorbic acid value being sometimes two to three times that originally present. No other substrate, including sodium D-glucuronate and sodium pyruvate, could be so converted into ascorbic acid. Subsequently, it was observed that L-gulonolactone, but not sodium L-gulonate, could also act as the substrate and this conversion took place in the absence of cyanide. Liver tissues of all the mammals investigated could bring about this cyanide-stimulated synthesis, but homogenates of the brain (rat), kidney (rat and goat) or adrenal gland (cow) were ineffective in this respect. Extracts from cow and goat livers were found to be the most active, and goat liver, being more freshly available, was chosen for further studies on the enzyme system concerned. The enzyme system was separated from goat-liver homogenates and obtained in the microsomes and the soluble fraction; it could be further concentrated from the extract by precipitation with ammonium sulphate (Chatterjee, Ghosh, Ghosh & Guha, 1958*a*). The present paper deals with detailed studies of this enzyme preparation.

EXPERIMENTAL

Enzyme preparation. The liver removed from the decapitated goat was immediately frozen and subsequently (within 3–4 hr.) homogenized in a Waring Blendor with 4 vol. of cold isotonic sucrose (with addition of 0·1 N-NaOH to keep the pH at 7·0) for 2 min., and centrifuged at 8500 *g* for 20 min. to sediment the nuclei and the mitochondrial fractions, following the method of Schneider & Hogeboom (1950). The supernatant, referred to in the text as the extract, was fractionally precipitated by addition of a saturated solution of ammonium sulphate at pH 6·0. The residue obtained at 30–50 % saturation of the salt was suspended in 10 mM-sodium phosphate buffer (pH 7·0) and was used after dialysis. It was subsequently found that by spinning at 100 000 *g* the entire activity was concentrated in the sedimented material; it is therefore not a soluble preparation. The temperature throughout the whole procedure was not allowed to rise above 0°.

Materials. Solutions of D-glucuronolactone, L-gulonolactone, potassium cyanide and other relatively unstable compounds used in the various experiments were freshly made and neutralized where necessary before addition to the reaction media. Sodium gulonate was prepared by incubating 17·8 mg. of L-gulonolactone with 1 ml. of 0·1 N-NaOH at 40° for 24 hr.

D-Glucuronolactone, sodium glucuronate, sodium galacturonate and D-gulono-γ-lactone were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.

Diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN) and adenosine triphosphate (ATP) were purchased from Schwartz Laboratories Inc., N.Y., U.S.A.

Incubation media. The test system contained: 20 mM-phosphate buffer (pH 7·0), 10 mM-substrate, 0·25 ml. of enzyme preparation (equivalent to 50 mg. of wet liver/ml. of incubation mixture) and 50 mM-KCN. Total volume was 2·5 ml.; incubation was for 2 hr. at 37° in air.

Estimation of ascorbic acid. This was carried out by adding 0·5 ml. of 30% metaphosphoric acid to 2·5 ml. of the incubated mixture, and filtering and titrating against 2:6-dichlorophenol-indophenol. When cyanide was not present in the reaction media, 'total' ascorbic acid was estimated according to the method of Roe & Kuether (1943). In the presence of reducing substances such as sodium dithionite and sodium hypophosphite Roe & Kuether's method was used with the modification that charcoal was replaced by bromine water for converting reduced ascorbic acid into dehydroascorbic acid. Whenever glutathione was present in the incubation mixture, the ascorbic acid was estimated titrimetrically according to the modified method of Mapson (1943).

Identification of the biosynthesized ascorbic acid. The ascorbic acid biosynthesized in the presence of cyanide has been identified by paper chromatography, following the method of Chen, Isherwood & Mapson (1953) with formic acid as the irrigating solvent. The presence of ascorbic acid was also tested by feeding the synthesized material to a scorbutic guinea pig. Because of the scarcity of material only one animal could be tested, but this showed a positive growth response to two doses of 1 mg. each of the biosynthesized material. Deproteinization and removal of cyanide were effected by adding conc. HCl (0·15 ml.) to the incubated medium (2·5 ml.), and centrifuging, and evaporating to dryness under suction and taking up the residue with water and neutralizing with sodium bicarbonate.

Estimation of protein. The estimation of the protein content of the homogenate, the extract and the enzyme preparation was carried out according to the method of Gornall, Bardawill & David (1949).

RESULTS

Activity of the enzyme preparation. The relative activities of the homogenate, the extract (containing the microsomes and the soluble supernatant) and the enzyme preparation are shown in Table 1. Unless otherwise mentioned, all the results represent those obtained after incubation of the enzyme preparation with D-glucuronolactone in the presence of potassium cyanide.

Effect of concentration of potassium cyanide on the synthesis of ascorbic acid. Fig. 1 shows that an increase in the cyanide concentration above 5 mM steadily increases the synthesis until a concentration of 50 mM is reached, when no further increase in the ascorbic acid value is observed. When the concentration is below 5 mM, practically no synthesis of ascorbic acid takes place.

Mode of action of cyanide. That cyanide actually facilitates the biosynthesis and does not merely act as an agent preventing the oxidation of ascorbic acid is revealed from the fact that cyanide at mM-concentration can completely protect the ascorbic acid added to the incubation media (Fig. 1), whereas at this concentration no biosynthesis is observed. When cyanide is replaced by other protectors of ascorbic acid, such as 8-hydroxyquinoline or sodium diethyl dithiocarbamate, no increase in the ascorbic acid value has been observed.

In order to throw further light on the mode of action of cyanide, the enzyme concentrate was preincubated with potassium cyanide in the absence of substrate and then dialysed to free it from cyanide. The enzyme concentrate, thus treated, could not convert D-glucuronolactone into L-ascorbic acid; the activity could be restored only after addition of potassium cyanide. Sodium cyanide with sodium phosphate buffer is equally as effective as potassium cyanide with potassium phosphate buffer.

Specificity of cyanide. No other metabolic inhibitor examined, e.g. 2:4-dinitrophenol, phlor-

Table 1. *Synthesis of ascorbic acid by the homogenate, the extract and the enzyme preparation*

Enzyme material	Ascorbic acid synthesized (μg./mg. of protein)	
	With cyanide	Without cyanide
Homogenate	7·0	0
Extract	10·5	0
Enzyme preparation	26·0	0

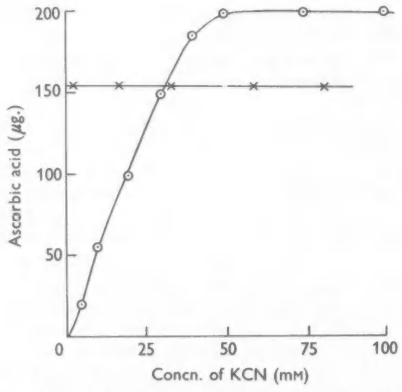

Fig. 1. Effect of cyanide concentration on the rate of conversion of D-glucuronolactone into L-ascorbic acid. ⊙, Influence of cyanide on the synthesis of ascorbic acid; ×, effect of cyanide on the protection of added ascorbic acid.

rhizin, Chloretone, each in mM-concentration, and sodium azide (mM, 50 mM), could replace cyanide for the conversion of D-glucuronolactone into L-ascorbic acid *in vitro*. In view of the possibility that cyanide might influence the biosynthesis by blocking an alternative metabolic pathway of D-glucuronolactone which might involve a cytochrome-linked enzyme system, incubation of D-glucuronolactone was carried out in a gas mixture of $CO + O_2$ (80:20) in the dark (and in the absence of cyanide) but without effect. When cyanide was replaced by reduced glutathione, sodium dithionite or sodium hypophosphite, each in a concentration of 50 mM, no increase in the ascorbic acid value was observed.

Specificity of substrate. With the exception of L-gulonolactone, no other substrate tested, e.g. sodium D-glucuronate, sodium L-gulonate, sodium D-galacturonate, D-gulono-γ-lactone, glucono-γ-lactone, sodium gluconate, sodium pyruvate, sodium pyruvate plus dihydroxyacetone or sodium citrate, was converted into L-ascorbic acid, all the substrates being used in 10 mM-concentration.

Table 2. *Synthesis of ascorbic acid from* D-*glucuronolactone,* L-*gulonolactone and the sodium salts of the corresponding free acids in the presence of cyanide*

Substrate	Ascorbic acid synthesized (μg.) Without cyanide	With cyanide
D-Glucuronolactone	0	210
Sodium glucuronate	0	0
L-Gulonolactone	450	188
L-Gulonate	0	0

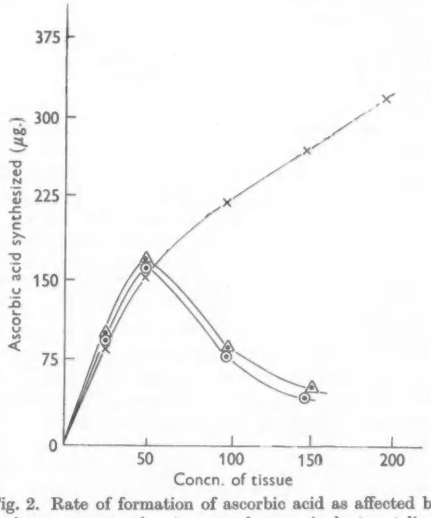

Fig. 2. Rate of formation of ascorbic acid as affected by tissue concentration (expressed as equivalent wet liver in mg./ml. of incubation mixture). ⊙, Homogenate; △, extract; ×, enzyme preparation.

The conversion of L-gulonolactone into L-ascorbic acid does not require the presence of cyanide and is, in fact, partially inhibited by it (Table 2).

Sodium glucuronate (10 mM) and sodium ascorbate (2–3 μmoles) did not exert any competitive inhibition on the conversion of D-glucuronolactone into ascorbic acid in the presence of cyanide.

Effect of variation of tissue concentration. It may be mentioned that under the influence of cyanide rat-liver slices could not convert D-glucuronolactone into L-ascorbic acid. It has also been noted that the homogenate as well as the extract contain some factors inhibitory to the synthesis of ascorbic acid under the conditions of the experiment, since it was found that the formation of ascorbic acid did not increase with increasing homogenate or extract concentration but rather decreased after a certain point (Fig. 2). But when the enzyme preparation was used instead, the synthesis became nearly proportional to the concentration of the former, the formation of ascorbic acid increasing with increasing enzyme concentration (Fig. 2). More recently it has been observed that the inhibitory factors are present in the supernatant fraction obtained after sedimentation of the liver extract at 88 700 *g* (Chatterjee, Ghosh, Ghosh & Guha, 1958*b*).

Other properties of the enzyme system. Treatment of the enzyme preparation with acetone or with alcohol (20%) or dilute acid (to pH 5·0), at 0°, resulted in inactivation of the enzyme system. When kept at −5°, it retained the full activity for about 2–3 weeks. When air was replaced by N_2 in the incubation media, no synthesis of ascorbic acid was observed either from D-glucuronolactone or L-gulonolactone. The enzyme concentrate, when dialysed against 10 mM-phosphate buffer at 0° even up to 60 hr., did not lose its activity for the conversion of D-glucuronolactone or L-gulonolactone. The optimum substrate concentration for the overall reaction was found to be 10 mM (Fig. 3), optimum pH was in the region of 6·5–7·0 (Fig. 4) and optimum temperature range was 37–42°. The maximum formation of ascorbic acid took place after an incubation period of 1·5 hr., after which no further increase was observed (Fig. 5).

Effect of metabolic inhibitors. The effects of some inhibitors on the cyanide-mediated biosynthesis were studied. Sodium azide, 2:4-dinitrophenol, phlorrhizin, arsenate, arsenite, pyrophosphate, each in mM-concentration, and sodium fluoride (up to 10 mM), were found to have no influence on

the conversion of D-glucuronolactone into L-ascorbic acid in the presence of cyanide.

Effect of thiol reagents. In studying the effect of some thiol reagents it has been found that mercuric chloride or *p*-chloromercuribenzoate, when used at a concentration of mM, could inhibit the synthesis to about 50%. Iodoacetate up to a concentration of 5 mM was without effect. Reduced glutathione (GSH), which was found incapable of further stimulating the effect of cyanide, could reverse the inhibition by *p*-chloromercuribenzoate to about 94% (Table 3). This indicates a possible involvement of some thiol group in the enzyme system catalysing the conversion of D-glucuronolactone into L-ascorbic acid *in vitro*.

Hassan & Lehninger (1956), however, observed that concentrations of GSH above 8 mM were significantly inhibitory to the rate of formation of ascorbic acid in their system. It is likely that the inhibition observed by them might be due to the fact that the method of Roe & Kuether (1943), which was used by them for the determination of 'total' ascorbic acid, would give low values for ascorbic acid in the presence of 5 mM and higher

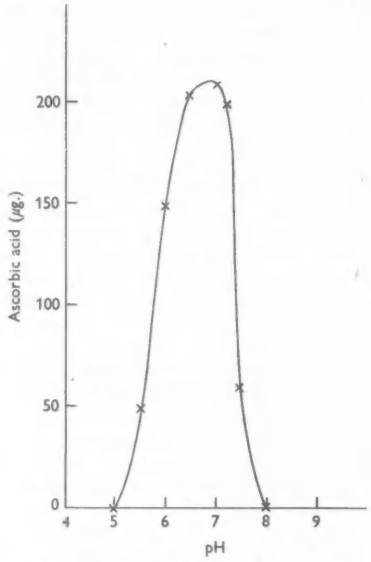

Fig. 4. Influence of pH on the enzymic conversion of D-glucuronolactone into L-ascorbic acid.

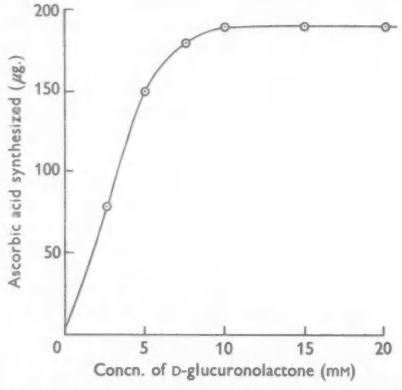

Fig. 3. Rate of formation of ascorbic acid as affected by substrate concentration.

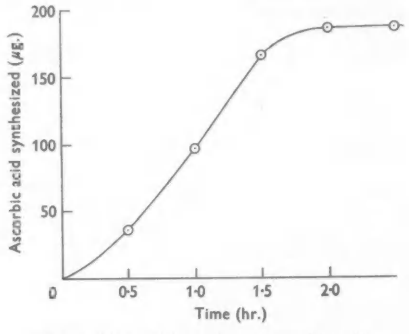

Fig. 5. Rate of formation of ascorbic acid.

Table 3. *Effect of thiol reagents on the synthesis of ascorbic acid from D-glucuronolactone* in vitro

Inhibitor	Concn. used (mM)	Ascorbic acid (μg.)	Inhibition (%)
None	—	200	—
Iodoacetate	5	200	0·0
Mercuric chloride	1	123	38·5
Mercuric chloride	5	0	100·0
p-Chloromercuribenzoate	1	88	56·0
p-Chloromercuribenzoate	5	0	100·0
GSH	10	200	0·0
p-Chloromercuribenzoate *plus* GSH	1 / 10	193	3·5

Table 4. *Inhibitory effects of adenosine triphosphate and uridine triphosphate on the biosynthesis of ascorbic acid* in vitro

ATP, adenosine triphosphate; UTP, uridine triphosphate.

Substrate	Inhibitor	Concn. used (mM)	Ascorbic acid (μg.)	Inhibition (%)
D-Glucuronolactone	None	—	228	—
	ATP	1	114	48
	UTP	1	89	61
L-Gulonolactone	None	—	180	—
	ATP	1	56	69
	UTP	1	36	74

concentrations of GSH. Thus in a recovery experiment starting with pure L-ascorbic acid it has been found that in the presence of 8 mM-GSH only 10–12 μg. out of 25 μg. of ascorbic acid could be estimated by that method.

Effect of other enzyme inhibitors. In studying the effects of other enzyme inhibitors it has been found that borate and Antabuse (disulfiram), each at mM-concentration, inhibited the cyanide-stimulated synthesis to about 50%. The inhibition by borate, however, was not reversed by sorbitol or ribose.

Effect of certain hydrogen acceptors. Dehydroascorbic acid (2–3 μmoles) or methylene blue (0·1 mM) had no influence on the rate of conversion of D-glucuronolactone into L-ascorbic acid in the presence of cyanide.

Requirement of cofactors

The fact that cyanide in a concentration of 50 mM, which can bind the DPN and TPN of reaction media (Colowick, Kaplan & Ciotti, 1951), could effect the biosynthesis indirectly indicates that DPN and TPN are not involved in this synthesis under the experimental conditions used. Moreover the liver extract, after treatment with active charcoal to free it from nucleotides (Stadtman, Novelli & Lipmann, 1951), retained its original activity (without any added cofactor) for the conversion of D-glucuronolactone into L-ascorbic acid.

It has also been found that in the presence of cyanide none of the cofactors examined, e.g. DPN, TPN, coenzyme A, glucose 1-phosphate, glucose 6-phosphate, reduced DPN, reduced TPN (generated *in situ* by addition of TPN, glucose 6-phosphate and glucose 6-phosphate dehydrogenase), each at mM concentration, $MgCl_2$ (5 mM), nicotinamide (30 mM), flavinadenine dinucleotide (0·1 mM) and flavinadenine mononucleotide (0·1 mM), was needed for the conversion of D-glucuronolactone into L-ascorbic acid by the dialysed enzyme concentrate *in vitro*. With L-gulonolactone, its conversion into L-ascorbic acid, as mentioned above, did not require any cyanide, and was not further enhanced by addition of DPN.

ATP and uridine triphosphate, on the other hand, when used at mM-concentrations in the incubation media, were found to have pronounced inhibitory effects on the rate of synthesis (Chatterjee, Ghosh, Ghosh, Roy & Guha, 1957*b*). This inhibition was not reversed by glucose and Mg^{2+} ions. No inhibition was observed with adenosine diphosphate (ADP), adenosine monophosphate, adenosine, adenine chloride or uridine diphosphate. The fact that the inhibition obtained was not due to the destruction of synthesized ascorbic acid in the presence of added ATP was revealed from a recovery experiment in which all of the ascorbic acid (2–5 μmoles), added to the tissue in the presence of ATP, could be estimated after an incubation period of 2 hr. The inhibitory effects of ATP and uridine triphosphate were also observed when D-glucuronolactone was substituted by L-gulonolactone as the substrate (Table 4).

DISCUSSION

The fact that D-glucuronolactone is converted into L-ascorbic acid in the presence of cyanide and that the conversion of L-gulonolactone into L-ascorbic acid takes place in the absence of cyanide indicates that the mechanism of action of cyanide relates to the reduction of D-glucuronolactone to L-gulonolactone. The overall synthesis of L-ascorbic acid from D-glucuronolactone is inhibited by *p*-chloromercuribenzoate, which effect is reversed by reduced glutathione. These results indicate that some –SH group or groups are probably involved in this reduction and that at least one of the functions of cyanide is to protect the –SH group from its oxidation to the disulphide linkage. Since the conversion of D-glucuronolactone into L-ascorbic acid is not inhibited by 2:4-dinitrophenol or sodium azide it would appear that phosphorylative reactions are not involved in the biosynthesis stimulated by cyanide.

There are apparently some fundamental differences between the animal and the plant systems in both the intracellular localization of the enzymes concerned and the nature of the enzyme systems involved. Mapson *et al.* (1954) have found that in

the plant system it is the mitochondria which catalyse the conversion of L-galactono-γ-lactone into L-ascorbic acid. This conversion probably involves the cytochrome system, as indicated by its inhibition with cyanide. In the rat liver, microsomes are the site of the enzyme system converting L-gulono-γ-lactone into L-ascorbic acid (Burns, Peyser & Moltz, 1956; Chatterjee *et al.* 1958*b*) and, as shown in this paper, the overall synthesis from D-glucuronolactone is activated by cyanide. There seems to be one point of similarity between the plant and the animal systems, namely, the inhibitory effect of ATP on the biosynthesis of ascorbic acid in the plant system and on the cyanide-stimulated biosynthesis with rat-liver tissue. The inhibition by ATP and uridine triphosphate may possibly be due to their promoting the decarboxylation of the substrate (Rabinowitz & Sall, 1957), which may be competitive with ascorbic acid formation.

That no added cofactor is needed for the conversion of D-glucuronolactone into L-ascorbic acid in the presence of cyanide, and that the dialysed enzyme retains the full activity, point to the possibility that the coenzymes required for the overall conversion in the reaction studied are firmly bound to the enzyme system concerned. Preliminary experiments indicate that flavoproteins may have some role in the oxidation–reduction system involved in the biosynthesis. All efforts, however, to resolve the enzyme system into apoenzyme and coenzyme have so far failed.

In the presence of cyanide, only the lactone form and not the sodium salt of the free acid is converted into ascorbic acid. Although ascorbic acid synthesis increases with enzyme concentration (Fig. 2) a high conversion has not been obtained. This has been traced to the fact that the enzyme preparation has a strong lactonase activity, rapidly hydrolysing the lactone to the free acid. It should be mentioned that in plant systems also only the lactone has been found to be the active precursor (Mapson *et al.* 1954). Using rat-liver extract Hassan & Lehninger (1956) have found that both the lactones and the sodium salts of D-glucuronic and L-gulonic acids are equally effective; addition of ATP and other cofactors is essential for this system. On the contrary, as reported in this paper, ATP has been found to be distinctly inhibitory to the conversion of D-glucuronolactone into L-ascorbic acid in the presence of cyanide. It is possible that there exist two separate pathways for the synthesis of ascorbic acid *in vitro*, one from the free acid requiring ATP and other cofactors, and catalysed by the enzyme system present in the supernatant as well as in the microsomes as suggested by Grollman & Lehninger (1957), and the other from the lactone only, which is stimulated by cyanide and requires no added cofactor. The latter enzyme system has recently been found to be localized entirely in the microsomes and is, in fact, inhibited by some factors present in the soluble supernatant (Chatterjee *et al.* 1958*b*). It has been observed that the addition of potassium cyanide to Hassan & Lehninger's system containing rat-liver microsomes, soluble supernatant and cofactors, further stimulates the synthesis of ascorbic acid from D-glucuronolactone (Chatterjee *et al.* 1957*a*). What exactly the relation is between the two systems will require further study.

SUMMARY

1. Potassium cyanide (50 mM), without any added cofactor, greatly stimulates the biosynthesis of L-ascorbic acid from D-glucuronolactone by an enzyme preparation from goat-liver extract containing the microsomes and the soluble supernatant.

2. The enzyme preparation can also convert L-gulonolactone into L-ascorbic acid with or without the presence of cyanide and in the absence of any added cofactor. But conversion is greater in the absence of cyanide.

3. The dialysed enzyme concentrate retains its full activity. Addition of cofactors such as di- or tri-phosphopyridine nucleotide, reduced di- or tri-phosphopyridine nucleotide, nicotinamide, magnesium chloride, coenzyme A, glucose 1-phosphate, glucose 6-phosphate and flavinadenine di- and mono-nucleotide does not further enhance the synthesis.

4. In the presence of cyanide only the lactone forms of the substrates are active. Neither the sodium salts of the free acids nor any other substrate tried are converted into ascorbic acid under the conditions studied.

5. The conversion of D-glucuronolactone is inhibited by *p*-chloromercuribenzoate and this inhibition is reversed by reduced glutathione, indicating the probability of the involvement of some thiol groups in the enzyme system concerned in the cyanide-mediated synthesis.

6. Adenosine triphosphate and uridine triphosphate have been found to be strikingly inhibitory to the synthesis of ascorbic acid from D-glucuronolactone and L-gulonolactone *in vitro* in the presence of cyanide.

7. The conversion of D-glucuronolactone into L-ascorbic acid in the presence of cyanide is not inhibited by sodium fluoride, sodium azide, 2:4-dinitrophenol, phlorrhizin, arsenite, arsenate or pyrophosphate.

8. Cyanide cannot be replaced by sodium azide, carbon monoxide and reducing substances such as reduced glutathione, sodium dithionite or sodium

hypophosphite for the conversion of D-glucuronolactone into L-ascorbic acid.

9. The presence of borate or Antabuse, each at mM-concentration, inhibits the conversion of D-glucuronolactone into L-ascorbic acid to about 50%.

10. The optimum conditions for the action of the enzyme system have been studied.

11. The biosynthesized ascorbic acid has been identified by paper chromatography and by a biological test with one guinea pig.

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Identification of 6-*O*-Acetyl-D-Glucopyranose in *Bacillus megaterium* Cultures: Synthesis of 6-*O*-Acetyl-D-Glucopyranose and 6-*O*-Acetyl-D-Galactopyranose

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This paper deals with the characterization of the crystalline carbohydrate obtained from *Bacillus megaterium* (NCIB 8508) cultures (Duff & Webley, 1958; and Duff, Webley & Farmer, 1957).

Preliminary examination (including infrared analysis and paper chromatography) showed that this compound was an ester of equimolar proportions of glucose and acetic acid. One carbonyl group per molecule was found with assays made at pH values within a narrow range around neutrality to avoid removal of a possible labile acetyl group. Separate experiments (Table 1) showed that little or no hydrolysis occurred at these pH values. From the above-mentioned evidence and because an acetylated phenylhydrazone and osazone were formed, it seemed unlikely that the hydroxyl groups on $C_{(1)}$ or $C_{(2)}$ were esterified. The failure to form a triphenylmethyl (trityl) ether strongly suggested that there was no free primary hydroxyl group. Helferich (1948) states that no case has been reported of a primary hydroxyl group unreactive towards trityl chloride with the conditions

used. The periodate oxidation of the ester was described previously (Duff *et al.* 1957; Duff, 1957); the failure to yield formaldehyde is almost certain proof that the primary alcoholic group is esterified.

An ester identical with that obtained from NCIB 8508 cultures can be prepared in the laboratory from glucose and 50% (v/v) acetic acid (Duff, 1957), but the process gives no information on the orientation of the acetyl group on the glucose molecule. It has now been possible to synthesize 6-*O*-acetyl-D-glucopyranose and the corresponding galactose ester via 6-*O*-acetyl-1:2-3:5-di-*O*-*iso*propylidene-D-glucofuranose and 6-*O*-acetyl-1:2-3:4-di-*O*-*iso*propylidene-D-galactopyranose respectively, thus confirming the structure suggested by analytical results (this paper and Duff, 1957). 3-*O*-Acetyl-D-glucose was also prepared, from 3-*O*-acetyl-1:2-5:6-di-*O*-*iso*propylideneglucofuranose.

Aqueous oxalic acid (0·2N) was found best for preferentially removing the *iso*propylidene group from the above intermediates but dilute mineral acid in ethanol also served. The 6-*O*-acetyl-D-galactose obtained is shown to be identical with the ester from galactose and 50% (v/v) acetic acid (Duff, 1957).

METHODS AND RESULTS

Melting points. These were determined with the Kofler micro-heating stage (Shandon Scientific Co., London).

Column chromatography. A slurry of Whatman cellulose powder (standard grade) and water-saturated butanol was packed into columns (5 cm. × 85 cm.) in small portions with a closely fitting ram perforated with small holes. The mixture was applied as a syrup with water or butanol or both and the column eluted with water-saturated butanol. Fractions (about 5 ml.) were collected with an automatic machine (Hough, Jones & Wadman, 1950). The distribution of the sugars in the tubes was found by paper chromatography with a spray of aniline phthalate or benzidine–trichloroacetic acid. The tubes were grouped and the solvent was removed at 30° *in vacuo*. In some cases the syrupy acetylated sugar crystallized, in others it was necessary to treat with charcoal in aqueous solution before crystallization. The products were dried to constant weight *in vacuo*.

Identification of the Bacillus megaterium *metabolite as* 6-O-*acetyl*-D-*glucopyranose*

Isolation. The crystalline *ester* was obtained as described by Duff & Webley (1958). After five recrystallizations from water it had m.p. 133°; $[\alpha]_D^{20}$ +42–48·2° at equilibrium in water (*c*, 4·0) (Found: C, 43·1; H, 6·3; $CH_3 \cdot CO$, 18·8. $C_8H_{14}O_7$ requires C, 43·2; H, 6·35; $CH_3 \cdot CO$, 19·4%). The substance separated from water as aggregates of minute prismatic rods. It was soluble in ethanol, acetic acid, ethyl acetate, pyridine and acetone, and insoluble in light petroleum (b.p. 80–100°) and in benzene.

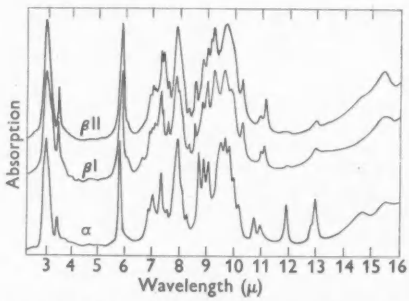

Fig. 1. Infrared spectra of different crystalline species of 6-*O*-acetyl-D-glucopyranose, isolated from *B. megaterium* cultures.

Occurrence of α- and β-anomers. The infrared-absorption spectra of several crystalline samples of the acetylglucose from *B. megaterium* cultures were examined, over the range 2–16 μ, with the potassium bromide pressed-disk technique (Schiedt & Reinwein, 1952). The spectra were recorded on a Grubb Parsons double-beam infrared spectrometer equipped with sodium chloride prism. The spectra showed some differences due to variation in the proportion of three crystalline species, one an α-anomer, and the others β-anomers (βI and βII) which probably differ only in their crystalline structures. The spectra of the purest available samples of these are given in Fig. 1. In most preparations one of the β-modifications predominated, giving typical β-glucopyranose bands in the 10·5–14 μ region (Barker, Bourne, Stephens & Whiffen, 1954) at 10·95 and 11·08 μ (913 and 903 cm.$^{-1}$) for the βI form, and at 10·92 and 11·11 μ (916 and 900 cm.$^{-1}$) for the βII form. The absorption spectrum of the α-anomer showed typical α-glucopyranose bands at 10·73, 10·96, 11·89, 12·82 and 12·96 μ (932, 912, 841, 780 and 771 cm.$^{-1}$). The spectrum of the α-anomer deviates widely from that of the two β-forms, which are clearly closely related although easily distinguished in the region 7–10 μ. In agreement with this interpretation, samples whose spectra indicated them to be nearly pure β-forms gave low initial rotations in water, about $[\alpha]_D$ +32°, whereas a sample of nearly pure α-anomer (m.p. 148–150°, obtained by recrystallizing from acetic acid as described by Reeves, Coulson, Hernandez & Blouin, 1957), gave an initial rotation of $[\alpha]_D^{14}$ +87·8° in water (*c*, 1·3). Mixtures of the α- and β-anomers gave inter-

mediate rotations. Spectra identical with the three forms of the natural acetylglucose were given by samples of the acetylglucose prepared from glucose and 50% (v/v) acetic acid (Duff, 1957). This supports the belief that the spectra correspond to three modifications of the same substance.

Absorption bands of the acetyl group (Bellamy, 1954) appear in all spectra. The band due to the C–O–C stretching vibration, at 7·87 μ in the β-anomers and 7·92 μ in the α-anomer, is at lower wavelengths than the composite band given by more fully acetylated sugars, which appears near 8·1 μ in the solid state (Kuhn, 1950), and at about 8·2 μ in CCl_4 solution, where it is associated with an inflexion or subsidiary maximum at 8·0 μ (Isbell *et al.* 1957). Fully acetylated sugars absorb at 7·3 μ in solution (Isbell *et al.* 1957) due to a deformation vibration of their methyl groups. There is a band at this wavelength in all three forms of 6-*O*-acetylglucose. The βII form has an equally strong band at 7·41 μ, which probably also arises from this deformation vibration by splitting due to the action of crystal forces. The carbonyl-stretching vibration of the acetyl groups, at 5·84 μ for the β-anomers and at 5·77 μ for the α-anomer, absorbs at longer wavelengths, owing to inter- or intramolecular hydrogen bonding, than does an unbonded ester carbonyl vibration which generally absorbs near 5·7 μ.

Examination of products of acid hydrolysis. The mixture obtained by hydrolysis (1·5N-H_2SO_4 for 4 hr. at 100°) was examined on the paper chromatogram after removal of acid with barium carbonate. The presence of glucose was indicated by its R_G value and was confirmed by epimerization with calcium hydroxide for 14 days and subsequent examination of the products by paper chromatography (Hough *et al.* 1950).

Glucosazone (m.p. 208° alone or mixed with authentic material) was obtained from another portion of the hydrolysate in a yield (49·6%) comparable with that obtained directly from an equivalent amount of glucose (46·5%).

To identify the acidic portion of the ester, hydrolysis was normally carried out with *p*-toluenesulphonic acid as in the quantitative method (Duff, 1957). In one experiment the volatile acid was neutralized exactly with 0·1N-NaOH and the solution evaporated to dryness. X-ray-powder photography of the residual solid showed that anhydrous sodium acetate was the only component. One spot only was obtained on a paper chromatogram prepared generally according to the method of Fewster & Hall (1951), and this was indistinguishable from the spot obtained by chromatography of sodium acetate. Duclaux numbers were determined generally as described by Morton (1938). The numbers, in this case based on the total acid collected in the first 10 portions (10 ml. each) of distillate, when the volume of solution originally distilled was 110 ml., were: 6·0, 7·0; 14, 14; 21, 22; 29, 29; 37, 38; 47, 48; 58, 57; 69, 68; 85, 81 for authentic acetic acid and for a sample of the volatile acid from acetylglucose respectively. The lack of agreement in the last numbers is probably due to unavoidable differences in the rate of heating with the simple apparatus used. Apart from this there seems no doubt that the volatile acid is acetic acid.

The glucose content of the ester was determined after hydrolysis with 1·5N-H_2SO_4 as previously described. With these conditions about 2% of the sugar is destroyed (Pirt & Whelan, 1951) so that the proportion of sugar indicated by a copper-reduction method (79·7%) agrees with the theoretical value (81·5%).

Reducing properties. The material showed reducing properties on a paper chromatogram with the aniline phthalate and ammoniacal silver nitrate reagents (Partridge, 1946, 1949).

Reduction with sodium borohydride by the method of Lemieux & Bauer (1953), and examination of the products on the paper chromatogram revealed a component with R_G value 0·28 (i.e. approximately the same as glucose acetate). This substance reacted with the borax–phenol red reagent for polyols (Hockenhull, 1953) but did not reduce ammoniacal silver nitrate nor form a coloured spot with aniline phthalate.

Determination of carbonyl by the Kiliani reaction. The quantitative application of the Kiliani reaction described by Frampton, Foley, Smith & Malone (1951) was used. The nitrile was formed in buffered solution at pH 8·5 and 7·5. At this latter value the reaction is only about 40% complete but there is less loss of the acetyl group by hydrolysis (Table 1). The ammonia obtained by alkaline hydrolysis of the nitrile was estimated.

At pH 8·5 glucose and acetylglucose gave 0·96 and 1·0 mol.prop., and at pH 7·5, 0·36 and 0·44 mol.prop. of –CHO respectively.

At pH 8·5 galactose and 6-*O*-acetylgalactose (Duff, 1957) gave 1·0 and 1·0 mol.prop. of –CHO respectively. Xylose and acetylxylose (Duff, 1957) gave 1·0 and 0·80 mol.prop. of –CHO respectively.

Determination of carbonyl by reaction with hydroxylamine hydrochloride. In this case the proportion of carbonyl in glucose and in acetylglucose was determined at the slightly acid conditions (pH 5·2) required for condensation with hydroxylamine. The determination was carried out generally as described by Gladding & Purves (1943). These authors recommend that the hydrochloric acid liberated in the buffered solution ($NH_2OH \cdot HCl$–NaOH) should be titrated with 0·5N-alkali, but it was found possible to use 0·13N-alkali with a

Table 1. *Hydrolysis of acetylglucose*

Acetylglucose (about 4 mg.) was dissolved in 0·1M-phosphate buffer or in acid (5 ml.) and the proportion of ester present determined by the colorimetric method initially and after a suitable period. The pH values were arranged to correspond with those used in the determination of carboxyl by the modified Kiliani reaction and with hydroxylamine, also with the preparation of the phenylosazone.

Medium	pH	Temp.	Time of hydrolysis (hr.)	Hydrolysis (%)
Buffer	8·5	37°	3	18
Buffer	7·5	37	3	0
Buffer	7·5	18	24	0
Buffer	5·2	100	1·5	13
Buffer	5·2	18	24	0
Buffer	3·1	100	0·3	0
N-H_2SO_4	—	28	4	50

corresponding increase in accuracy when the solution was continuously stirred and the end point (at pH 3·2) was indicated by a pH meter and glass electrode.

After 18 hr. glucose (0·1402 g.) and acetylglucose (0·1671 g.) liberated acid corresponding to 1·11 and 1·15 mol.prop. of –CHO respectively. Galactose and 6-*O*-acetylgalactose (Duff, 1957) gave 0·95 and 0·95 mol.prop. of –CHO respectively. Xylose and acetylxylose (Duff, 1957) gave 0·95 and 1·1 mol.prop. of –CHO respectively.

Formation of a phenylhydrazone. Distilled phenylhydrazine (0·56 g., 1 mol.prop.) in methanol (10 ml.) was added to acetylglucose (1 g.) and the *phenylhydrazone* isolated in the usual way and recrystallized from methanol: m.p. 134–136°; $[\alpha]_D^{20} - 13°$ in water (*c*, 1·3) (Found: $CH_3 \cdot CO$, 12·9; N, 8·60. $C_{14}H_{20}O_6N_2$ requires $CH_3 \cdot CO$, 13·8; N, 8·97 %).

Formation of a phenylosazone. When heated for 30 min. with a solution of phenylhydrazine acetate buffered at pH 5·8 only glucosazone was obtained (in small yield). The procedure of Levene, Meyer & Raymond (1931) gave about 25 % of the theoretical yield of an *osazone* (Found: $CH_3 \cdot CO$, 9·9. $C_{20}H_{24}O_5N_4$ requires $CH_3 \cdot CO$, 10·8 %) with an infrared spectrum very similar to that of glucosazone but with an additional band at 5·79 μ corresponding to an acetyl carbonyl group.

Attempted preparation of a trityl ether. The method of Reynolds & Evans (1938) was used. Several experiments were performed with 1–3 g. amounts of ester or glucose and 1·1 mol.prop. of trityl chloride in dry pyridine followed by acetic anhydride. With glucose a reasonable yield of crystalline 1:2:3:4-tetra-*O*-acetyl-6-*O*-trityl-β-D-glucose was obtained but in no case was a trityl derivative obtained from acetylglucose.

Synthesis of 6-O-acetyl-D-glucopyranose

6-O-*Acetyl*-1:2-*iso*propylidene *glucose.* This was prepared by the method of Bell (1936) via the 1:2-*iso*propylidene-3:5-boric ester. Direct hydrolysis of the former substance with 0·5N-sulphuric acid was unsatisfactory in that the 6-*O*-acetyl-D-glycopyranose obtained (in poor yield) did not crystallize although it was chromatographically identical with the natural ester.

6-O-*Acetyl*-1:2-3:5-di-O-iso*propylidene glucose.* This was prepared from the mono-*iso*propylidene derivative (2·04 g.) by the method of Macphillamy & Elderfield (1939). The syrupy product (2·25 g.) was distilled twice in high vacuum (0·005 mm. Hg) at 140° and had $[\alpha]_D^{18} + 36°$ in $CHCl_3$ (*c*, 1·03) [Found: $(CH_3)_2 \cdot C$, 31·0; $CH_3 \cdot CO$, 12·0. Calc. for $C_{14}H_{21}O_7$, $(CH_3)_2 \cdot C$, 27·9; $CH_3 \cdot CO$, 14·2 %]. Ohle & Varga (1929) recorded $[\alpha]_D + 33°$ for their product, obtained via 1:2-3:5-di-*O*-*iso*propylidene 6-*O*-*p*-toluenesulphonyl-D-glucose. Natural 6-*O*-acetyl-D-glucose (1 g.) was converted into the di-*O*-*iso*propylidene derivative (1·2 g.) similarly. The syrup obtained had $[\alpha]_D^{18} + 29·0$ in $CHCl_3$ (*c*, 1·08). Its infrared spectrum (capillary film) differed from that of the synthetic material only in having additional absorption at 13·19 and 11·49 μ, presumably due to an impurity.

6-O-*Acetyl*-D-*glucopyranose.* 6-*O*-Acetyl-1:2-3:5-di-*O*-*iso*propylidene-D-glucose (0·4 g.) in ethanolic solution was treated with 10 ml. and then 20 ml. of 0·1N-H_2SO_4 and left at room temperature for 3 hr. and then 6 hr. Examination by the paper chromatograms (benzidine–trichloroacetic acid spray) showed that the material had not been hydrolysed by these treatments. Accordingly the acid concentration was raised to 0·5N by the addition of 2N-acid. After 80 hr. at room temperature sulphuric acid was removed with barium carbonate and the supernatant solution evaporated to a syrup *in vacuo* at 25°. Chromatography on a cellulose column (40 cm. × 2·0 cm.) gave bulked fractions containing 14·6 and 36·4 mg. of a mixture (acetylglucose–glucose) and of acetylglucose respectively. The remaining fractions contained mono- and di-*O*-*iso*propylidene-6-*O*-acetylglucose. The fraction containing only acetylglucose crystallized and had $[\alpha]_D^{14} + 47·6$ at equilibrium in water (*c*, 1·3) in good agreement with the value obtained for the natural ester. The infrared spectrum (Fig. 2) was virtually identical with that of the natural material, in the βI form (Fig. 1). The R_g values of the natural and synthetic material were identical when run in four different solvent systems [butanol saturated with water, butanol–acetic acid–water (4:1:5, by vol.), phenol–ethanol–water (3:1:1, w/v/v), and ethyl acetate–acetic acid–water (3:2:3, by vol.)].

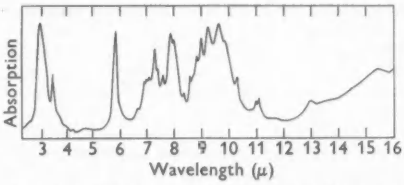

Fig. 2. Infrared spectrum of 6-*O*-acetyl-D-glucopyranose prepared from 6-*O*-acetyl 1:2-3:5-di-*O*-*iso*propylidene-D-glucose.

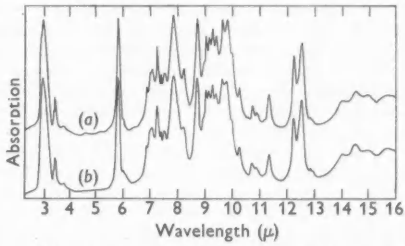

Fig. 3. Infrared spectra of 6-*O*-acetyl-D-galactopyranose: (*a*) prepared by acetylation of galactose with 50% acetic acid; (*b*) prepared from 6-*O*-acetyl-1:2-3:4-di-*O*-*iso*propylidene-D-galactose.

Synthesis of 6-O-*acetyl*-D-*galactopyranose*

Acetylation of diacetone galactose with sodium in dry ether followed by acetyl chloride (cf. Freudenberg & Smeykal, 1926) gave 6-*O*-acetyl-1:2-3:4-di-*O*-*iso*propylidene-D-galactose (Ohle & Berend, 1925). The *iso*propylidene groups from this (2·36 g.) were removed by heating at 55° with oxalic acid in 50% ethanol (0·2N, 1250 ml.) for 3 hr. (modified from the method of Levene & Tipson, 1936). Oxalic acid was removed with calcium carbonate and the filtrate evaporated *in vacuo* at 25° to a syrup. Paper chromatography showed that galactose, acetylgalactose and mono-*iso*propylidene acetylgalactose were the main components. Separation on a cellulose column gave a crystalline bulked fraction (0·34 g.) containing only 6-*O*-acetyl-D-galactose. The infrared spectrum did not clearly indicate a pyranose structure (Barker *et al.* 1954; Barker & Stephens, 1954), but such a structure seems likely from the method of preparation. The ester had m.p. 132–135° alone or mixed with material prepared by preferential acetylation of galactose with 50% (v/v) acetic acid (Duff, 1957), and the infrared spectra were virtually identical (Fig. 3). The R_G values on the paper chromatogram (0·25) were identical: $[\alpha]_D^{14}$ +66·9° at equilibrium in water (*c*, 5·1) (Found: $CH_3 \cdot CO$, 19·3; $C_8H_{14}O_7$ requires $CH_3 \cdot CO$, 19·4%).

Synthesis of 3-O-*acetyl*-D-*glucose*

3-*O*-Acetyl-1:2-5:6-di-*O*-*iso*propylidene-D-glucose (4·25 g., prepared generally according to Fischer & Noth, 1918) was hydrolysed as usual with 0·2N-oxalic acid (2000 ml.). After separation on a cellulose column (65 cm. × 2·6 cm.) a glass (0·37 g.) was obtained. This had $[\alpha]_D^{14}$ +46° at equilibrium in water (*c*, 4·3). This gave a single spot with moderate 'trailing' when applied directly to the chromatogram (R_G 0·33), but on keeping in water an isomerization occurred and two spots, R_F 0·28 and 0·33, were obtained in approximately equal concentration. The former spot may correspond to 6-*O*-acetylglucose formed by transfer of an acetyl group via an ortho ester as recorded by Josephson (1929), who found that 3-*O*-acetyl-1:2-*iso*propylidene-D-glucose was converted into the 6-*O*-acetyl derivative when held in solution.

SUMMARY

1. The ester isolated from cultures of *Bacillus megaterium* (NCIB 8508) has been characterized as 6-*O*-acetyl-D-glucopyranose because of the following properties: (*a*) It has reducing properties and forms a phenylhydrazone. (*b*) It has 1 mol. prop. of –CHO (determinations were carried out on either side of pH 7·0 by different methods). (*c*) It forms a phenylosazone. (*d*) It does not form a trityl ether.

2. The hydrolysis of 6-*O*-acetyl-D-glucopyranose is described under various conditions of pH and temperature.

3. 6-*O*-Acetyl-D-glucopyranose and the corresponding galactose ester have been synthesized by methods designed to illustrate their structure, i.e. via the known di-*O*-*iso*propylidene ethers of the parent sugars.

4. The unstable syrupy 3-*O*-acetyl-D-glucose is similarly prepared.

We wish to thank Mr W. A. Mitchell of this Institute for taking and interpreting the X-ray-powder photograph. Mr C. M. Mundie provided skilled technical assistance.

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Metabolism of 6-*O*-Acetyl-D-Glucopyranose and other Monoacetyl-sugars by Strains of *Bacillus megaterium* and other Soil Organisms

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The discovery that 6-*O*-acetyl-D-glucopyranose is a major metabolite of a cobalamin-producing strain of *Bacillus megaterium* (NCIB 8508) has been reported (Duff, Webley & Farmer, 1957). The present paper describes the production of the ester by growing and resting cells of this strain (and others) and the occurrence of various enzyme systems in cell-free extracts, including a specific 6-*O*-acetyl-D-glucose deacetylase.

The production of an acetylated sugar by an organism that synthesizes cobalamin is of particular interest in that the vitamin itself contains acetyl and ribose residues. Cobalamin production has been reported in 10 of the 14 strains of *B. megaterium* used in this work (Garibaldi, Ijichi, Snell & Lewis, 1953), but only three produced acetylglucose. The strain NCIB 8508, however, produced the greatest amount of both metabolites. Acetylglucose is a major product of this strain, as up to 25% of the glucose used appears as the ester in growth experiments.

This work represents the first isolation of an acetylated monosaccharide from natural sources although acetylated polysaccharides have been found in nature (e.g. in the gum from *Sterculia setigera*: Hirst, Hough & Jones, 1949).

MATERIALS AND METHODS

Organisms. The cobalamin-producing strain of *B. megaterium* mainly used was obtained from the National Collection of Industrial Bacteria (NCIB 8508). A full description of its characteristics is given by Garibaldi *et al.* (1953). It is referred to by these workers as *B. megaterium* NRRL B-938. Also tested were two other strains of *B. megaterium* from the NCIB (2605 and 7581), and 11 from Dr N. R. Smith's collection (NRS strains) obtained by courtesy of Dr Ruth Gordon, Institute of Microbiology, Rutgers University, U.S.A.

The cultures were maintained as spores formed after growth on nutrient-agar slopes. These stocks were kept at 2° and cultures were restarted from them when required by subculture on to glucose–nutrient-agar slopes or plates.

Unless otherwise stated, the strain numbers in the text refer to the NCIB strains of *B. megaterium*.

Large-scale growth experiments. These were carried out on a shaking machine (Webley & Duff, 1955) in a constant temperature room at 25° in 1 l. Pyrex culture flasks (no. 1410), each containing 200 ml. of liquid medium. The composition of the medium used, unless otherwise stated, was as follows: glucose, 50 g.; KH_2PO_4, 0·5 g.; K_2HPO_4, 0·5 g.; $MgSO_4,7H_2O$, 0·5 g.; KCl, 0·2 g.; $FeSO_4$, 0·02 g.; $CoCl_2,6H_2O$, 0·012 g.; $(NH_4)_2HPO_4$, 1 g.; yeast extract (Difco), 2 g.; $CaCO_3$, 10 g.; 1 l. of water. The $CaCO_3$ was sterilized separately in the culture flasks with 5 ml. of water. The mineral salts + yeast extract and the glucose

were also sterilized separately and then added aseptically to the culture flask when all had cooled. This was done to prevent loss of ammonia and formation of insoluble phosphates during autoclaving. Autoclaving was at 22 lb./in.^{2} for 20 min. Each flask was inoculated with 5 ml. of a thick suspension prepared from an 18 hr. growth on a glucose–nutrient-agar slope.

Small-scale growth experiments. These were performed with 10 ml. of the medium described above in 4 oz. flat bottles but with 2% (w/v) glucose and 0·4% $(NH_4)_2HPO_4$. The $CaCO_3$ (0·1 g.) was autoclaved with 1 ml. of water in the bottles. The mineral salts + yeast extract and substrates were sterilized separately by autoclaving or bacterial filtration and added as described above. Each bottle was inoculated with two drops of a thick suspension prepared as described above. After inoculation the bottles were attached to the shaking machine and examined daily for growth.

Production and preparation of cells for resting-cell experiments. Plates of glucose–nutrient agar were heavily seeded from an 18 hr. growth on glucose–nutrient agar. Heavy growth was obtained by spreading the inocula over the surfaces of the plates with a sterile bent glass rod. The plates were incubated at 25° overnight (about 17 hr.). The cells were washed off the plates next morning with Sörensen 0·1 M-phosphate buffer, pH 7·0, and washed once with the buffer in the centrifuge. They were finally resuspended in the buffer and the dry weight was determined turbidimetrically. Although rather wide variations in activity were noted from different batches of cells the (qualitative) conclusions drawn from the experiments are not affected. Each experiment was performed at least twice with different batches of cells.

Preparation of cell-free extracts. The organism was grown on plates as described above, and washed off with buffer solution (as above or with 0·02 M-veronal buffer, pH 7·2, when the extract was used for the acidimetric method of Glick, 1949) and harvested by centrifuging at 35 000 *g* for 1 hr. in an MSE Super-Speed 20 refrigerated centrifuge. Grinding was carried out as described by McIlwain (1948) but with H Fine Aloxite (Carborundum Co. Ltd., Manchester) four times the wet weight of the cells. After removing most of the alumina at 5000 rev./min. the extracts were clarified at 50 000 *g* for 1 hr. in the centrifuge mentioned above. In some cases cell-free extracts were obtained by crushing in a press (Hughes, 1951) or by treating with lysozyme (Weibull, 1953). The method of Stadtman, Novelli & Lipmann (1951) was used to estimate and adjust the protein content of the extracts. Crystalline egg albumin and bovine plasma albumin (90% protein; obtained from Armour and Co. Ltd., Eastbourne, Sussex) were used as standards.

Chromatography and the estimation of acetic acid. These were carried out as described by Duff (1957).

Acetylglucose. The colorimetric ester assay of Hestrin (1949) was used. With resting cells and with high concentrations of substrate (e.g. pyruvate) rather high blanks were obtained by this method and paper chromatography was preferred. The papers were spotted quantitatively and the spots obtained sprayed with aniline phthalate and compared with standards with a Recording Double-beam Reflectance Densitometer (Joyce-Loebl Ltd., Vine Lane, Northumberland Street, Newcastle upon Tyne 1).

Lactic acid. This was determined by a method based on that of Friedemann & Graeser (1933).

Glucose. The Somogyi (1945) method was used for determination of glucose.

Acetylphosphate. The dilithium salt was prepared with *iso*propenyl acetate and phosphoric acid by the method of Stadtman & Lipmann (1950).

Standardization was carried out by the method of Lipmann & Tuttle (1945) with succinic anhydride.

Bacterial filtration. In some cases (e.g. Table 3) it was convenient to remove cells by using a Hemmings-type bacterial filter. This was obtained from Mr H. A. Jones, 26 Castle Street, Beaumaris, Anglesey.

Buffer solution. Unless otherwise indicated Sörensen 0·1 M-phosphate buffer, pH 7·0, was used.

Enzymes and coenzymes

Taka-diastase, adenosine triphosphate (ATP) and lipase (source unknown) were obtained from L. Light and Co. Ltd., Colnbrook, Bucks, pancreatin from British Drug Houses Ltd., Poole, Dorset, crystalline lysozyme from Armour and Co. Ltd., Eastbourne, Sussex, glucose oxidase and coenzyme A (75%) from the Sigma Chemical Co. (G. T. Gurr Ltd., 136 New Kings Road, London, S.W. 6). A sample of coenzyme A (15%) was kindly provided by Dr E. M. Crook, Biochemistry Department, University College, London.

RESULTS

Production of acetylglucose by growing organisms

A series of small-scale growth experiments was set up to investigate the production of acetylglucose. Strains 8508, 7581 and 2605 and 11 NRS strains were used. Other organisms included strains of *Bacillus polymyxa*, *Bacillus licheniformis*, *Aerobacter aerogenes* (four strains), *Agrobacterium radicola*, *Nocardia opaca* (strain T_{16}) and six unidentified gum-producing bacteria (including sporing and non-sporing rods and a pleomorphic organism) isolated from soils by Forsyth & Webley (1949).

Samples were taken from each bottle at intervals (usually 2 days) until the substrate was exhausted. The ester formed was estimated by quantitative paper chromatography. Strains 8508, 7581 and NRS 697 gave 2·75, 0·37 and 0·35 mg. of acetylglucose/ml. No ester could be detected in the other samples.

Isolation of acetylglucose from liquid cultures of strain 8508

Usually five or six 1 l. flasks with 200 ml. each of the medium (containing 4% of glucose), after inoculation, were shaken on the machine in a constant-temperature room at 25° and small samples were removed aseptically at convenient intervals for colorimetric determination of the acetylglucose formed. At the time of maximal ester production the contents of the flasks were combined and calcium carbonate and cells removed with an Alfa-Laval type 1229 centrifuge (Alfa-Laval Co. Ltd., Brentford, Middlesex). An equal volume of 95% ethanol was added to remove polysaccharides. After evaporation to small bulk the

addition of ethanol was repeated and the supernatant solution was evaporated at 30° *in vacuo*. The remaining aqueous solution (100–200 ml.) was passed through columns (2·5 cm. × 40 cm.) of Amberlite ion-exchange resins (IR 120H and IR 4B in that order) and concentrated to a syrup at 30° *in vacuo*. The syrup was added to a large column (5 cm. × 85 cm.) of powdered cellulose and a fraction containing acetylglucose alone obtained in the usual manner (Duff & Farmer, 1958). Butanol was removed *in vacuo* and the syrupy product set aside to crystallize. The crude material was recrystallized by dissolving it in water at 40–50° and setting aside in an evacuated desiccator over anhydrous calcium chloride. The paste of syrup and crystals was then separated on a porous tile. It was now found possible to recrystallize the compound from ethanol.

In a typical growth experiment, after incubation for 4 days, 1200 ml. of 4% glucose medium gave 11·8 g. of crystalline acetylglucose. The colorimetric method had indicated a total ester production of about 25 g. In addition to mechanical losses some hydrolysis would be expected on the strongly acid IR 120H resin column and, as indicated later, a small proportion of glucose esters of other low-molecular-weight fatty acids are formed.

Variation of ester and acid production with time and substrate concentration during growth of strain 8508

Fig. 1 gives details of a growth experiment set up to investigate the production and disappearance of both acetylglucose and total volatile acid. These follow the same general course during the growth period. The exhaustion of substrate (shown by paper chromatography) corresponds with the greatest concentrations obtained of both acetylglucose and total volatile acid. Acetylglucose was determined colorimetrically with 1 ml. samples. It was not possible directly to determine the free volatile acid produced in the presence of acetylglucose but this was obtained by difference.

The total volatile acid (i.e. free acid plus that from hydrolysis of acetylglucose) was estimated on 5 ml. samples. Examination of the curves shows that about 2·6 and 3·4 m-moles/200 ml. (of acetylglucose and free acetic acid respectively) were produced at the time when glucose was exhausted.

It was shown that acetic acid was the only volatile acid produced in quantity. In a separate growth experiment with similar quantities of substrate and medium the cells were harvested by centrifuging and a portion of the supernatant was hydrolysed. The volatile acid was separated by steam-distillation and neutralized exactly with sodium hydroxide solution and an external indicator. Examination by paper chromatography

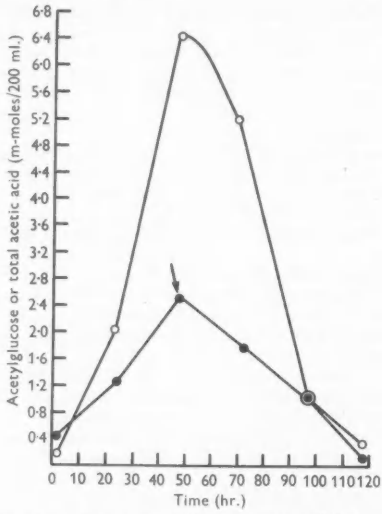


Fig. 1. Production and utilization of acetylglucose and acetic acid by growing cells of strain 8508 on 2% (w/v) glucose media. ●, Acetylglucose; ○, acetic acid. The arrow indicates the time at which glucose disappeared from the medium (about 45 hr.). Total acetic acid is the sum of the free acid and that liberated by hydrolysis of acetylglucose in the course of the determination.

(Fewster & Hall, 1951) showed that the main product was indistinguishable from sodium acetate. Traces of higher acids (butyric acid and an unidentified acid) were also observed.

Table 1 shows the effect of increasing concentrations of substrate (and ammonium phosphate) on the production of acetylglucose. The ester (determined colorimetrically) accumulated to a concentration of about 12 mg./ml. with 4% (w/v) glucose.

Production of ester by strain 8508 *with substrates other than glucose*

A series of small-scale growth experiments was set up with other common carbohydrate substrates and the ester formed followed at suitable intervals (colorimetrically and by paper chromatography). Mannose, galactose, arabinose, xylose, glucose 1- and 6-phosphate, glyceraldehyde, maltose and fructose did not produce any esters. Growth occurred on all sugars except glyceraldehyde. As expected, a large amount of ester was produced from sucrose and this could not be distinguished from acetylglucose on the paper chromatogram. Glucose gave a yield of 2·9 mg. of acetylglucose/ml. and sucrose produced 2·36 mg. of ester/ml. (as acetylglucose) under identical conditions.

Table 1. *Effect of varying concentration of substrate on 6-O-acetylglucose production by strain* 8508

Three culture flasks were set up with mineral salts and calcium carbonate (final vol. 200 ml.). The flasks contained 1, 2 and 4% (w/v) of glucose and 0·2, 0·4 and 0·8% of ammonium phosphate respectively. After inoculation the flasks were incubated at 28° with shaking and samples withdrawn aseptically for acetylglucose determination by the colorimetric method.

Concn. of glucose (%, w/v)	Acetylglucose formed (mg./ml.) after incubation for							
	18 hr.	24 hr.	42 hr.	48 hr.	66 hr.	72 hr.	90 hr.	144 hr.
1	0·16	0·82	0·78	0·78	0·42	0·38	—	—
2	0·27	1·3	3·7	3·7	3·7	3·1	—	—
4	0·15	1·0	3·2	4·6	8·5	10·5	12·2	7·1

Other metabolic products of strain 8508

Lactic acid. Cells were grown on the medium (100 ml.) but containing only 0·5% of glucose and 0·1% of ammonium phosphate. Samples (10 ml.) were removed at intervals and the remaining glucose and the lactic acid determined in the supernatant. In 48 hr. the glucose concentration had fallen to 0·13–0·20% (two experiments). There was no lactic acid present. With resting cells (final concentration 5 mg./ml.) similar results were obtained, the initial glucose concentration of 0·51% falling to 0·34% after shaking for 6 hr. in an atmosphere of oxygen. During that period 0·09 mg. of lactic acid/5 ml. was produced, representing only 0·1% of the glucose used.

Amino acids. Table 2 shows that pyruvate is partly metabolized to α-alanine and glutamic acid in the presence of ammonium phosphate.

Table 2. *Conversion of pyruvic acid into alanine and glutamic acid by resting cells of strain* 8508

Thunberg tubes were set up with pyruvic acid (1·5 m-moles as sodium salt in 0·5 ml.) or 0·1 M-phosphate buffer, pH 7·0 (0·5 ml.), ammonium phosphate (0·4 m-mole in 0·5 ml.) or buffer (0·5 ml.) and cell suspension (1 ml. containing 30 mg. dry wt.). The tubes were shaken in air for 6 hr. at 28°. After removing the cells by centrifuging, the supernatant solutions were passed through columns (1 cm. × 20 cm.) of Zeo-Karb 225 in the H^+ form. The solution emerging and water washings (10 ml.) of the column did not contain amino acids. The columns were eluted with aq. 10% (w/v) NH_3 soln. (50 ml.) and the eluates evaporated to dryness. Water (1 ml.) was added to the residues and the amino acids were determined quantitatively on a one-dimensional paper chromatogram with phenol–ethanol–water (3:1:1, w/v/v) as solvent and with the Joyce-Loebl recording double-beam reflectance densitometer (Dr R. I. Morrison of this Institute was responsible for these analyses.)

System	Amino acids formed (μmoles)		
	α-Alanine	β-Alanine	Glutamic acid
Cells alone	1·6	Nil	3·1
Cells + pyruvate	2·2	0·3	8·5
Cells + pyruvate + ammonium phosphate	3·1	Nil	27

Production and hydrolysis of acetylglucose by resting cells

Effect of various additions on the synthesis of acetylglucose by resting cells of strain 8508. This is shown in Table 3. No synthesis of the ester occurred when the cell suspension was boiled for 1 min. The results show that pyruvate and acetate but not citrate exert an initial stimulatory effect on ester production. This effect was not apparent in the 6 hr. samples when, presumably, glucose had broken down to supply sufficient 'active acetate' to swamp the effect of the additions. A similar experiment was reported previously (Duff *et al.* 1957), where the more specific determination of acetylglucose by paper chromatography was used. A further experiment (Table 4) showed the effect of oxygen and nitrogen on ester synthesis from glucose alone and with added sources of active acetate. Similarly, experiments were set up with glucose and other substrates. After incubation for 5 hr. glucose, glucosamine, galactose and fructose produced 50, 14, 5 and 5 μmoles of ester as estimated by the colorimetric method. Gluconate, glucose 1-phosphate (potassium salt), ribose, mannose, choline, xylose and fructose 6-phosphate (calcium salt) gave no esters. On the paper chromatogram spots corresponding to esters were obtained only with glucose and glucosamine. (The R_g of the ester from the latter was 0·16, cf. *N*-acetylglucosamine 0·18 and 6-*O*-acetylglucose 0·28.) With *B. megaterium* strains NRS 697, NCIB 2605 and *Lactobacillus plantarum* NCIB 7220 (grown as described by Stephenson & Rowatt, 1947) only the first-mentioned produced the ester (4·6 μmoles at 6 hr.; cf. Table 3).

Effect of inhibitors. Thunberg tubes were used containing cell suspensions in 0·1 M-phosphate buffer (1 ml.; 46 mg. dry wt.), glucose solution (1 ml. containing 222 μmoles) and inhibitor (0·1 ml. of a suitable concentration in water) or water (0·1 ml.). After incubation for 4 hr. in oxygen in the usual way, the cells were removed with a bacterial filter and the ester concentration was determined in a portion of the filtrate by the colorimetric method. Glucose alone and in the presence

of sodium arsenite (5 mM), sodium fluoride (2 mM), sodium azide (mM), sodium cyanide (mM), sodium iodoacetate (5 mM), sodium *p*-chloromercuribenzoate (mM), phlorrhizin (50 mM), urethane (50 mM) or semicarbazide (20 mM) gave 12, 7·6, 8·4, 9·8, 10·8, 8·6, 8·5, 10·0, 11·7 or 9·4 μmoles of acetylglucose respectively. The generally insensitive nature of the synthesis may be due to poor penetration of the inhibitors into the capsulated cell.

Table 5 shows the effect of sodium arsenite on the accumulation of pyruvic acid during ester synthesis in oxygen and in nitrogen. The acid was determined by the method of Friedemann & Haugen (1943). The sample and the hydrazine reagent were incubated for 25 min. and the hydrazone was extracted with ethyl acetate (8 ml.), so that the figures represent total keto acid concentration. However, the infrared spectra of the hydrazones from a similar experiment with 2 mM-arsenite showed that the product was practically pure pyruvic acid 2:4-dinitrophenylhydrazone. Paper chromatography (Isherwood & Cruickshank, 1954) confirmed this and indicated that a small proportion of α-oxoglutaric acid 2:4-dinitrophenylhydrazone was obtained from a similar system with mM-arsenite.

Hydrolysis of acetylglucose. A comparison was made of the breakdown of samples of 6-*O*-acetylglucose isolated from strain 8508 cultures and others prepared synthetically (Duff, 1957). The esters with water (1·5 ml.) and cell suspension in buffer (1·5 ml.; 34 mg. dry wt./ml.) were shaken in Thunberg tubes in an atmosphere of oxygen at 28°. Samples (0·25 ml.) were removed at intervals and the ester concentration was determined by the colorimetric method. The 'natural' and 'synthetic' ester present was respectively 16·0 and 15·6 μmoles (0·25 hr.); 13·0 and 13·4 μmoles (2 hr.); 13·0 and 13·4 μmoles (4 hr.); 4·7 and 6·4 μmoles (18 hr.). In a second experiment, the more specific determination of ester by paper chromatography being used, similar results were obtained, except that no ester survived the overnight incubation. Glucose did not appear in any of the samples, showing that the sugar was metabolized at least as quickly as the ester was hydrolysed.

Table 3. *Effect of pyruvate, citrate and acetate on the synthesis of* 6-O-*acetylglucose from glucose by resting cells of strain* 8508

Filter flasks (100 ml.) were charged with glucose (0·89 m-mole) in water (2 ml.), washed-cell suspension (4 ml., 41 mg. dry wt./ml., in 0·1 M-phosphate buffer, pH 7·0) and buffer (2 ml.) or sodium pyruvate, citrate or acetate (1·45 m-moles in buffer, 2 ml.). Each flask was exhausted, charged with oxygen through the side arm and shaken in a thermostatic bath at 28°. Samples were removed at intervals and cells removed by centrifuging or by passing through a bacterial filter (Hemmings type). Acetylglucose was estimated by the colorimetric method. Flasks were refilled with oxygen after removing each sample. pH remained unchanged at the initial value of 7·0 throughout the experiment.

Time (hr.)	6-*O*-Acetylglucose* formed (μmoles)			
	Glucose only	Glucose + pyruvate	Glucose + citrate	Glucose + acetate
2	16	20	16	23
4	29	36	28	38
6	40	37	42	35

* Corrected for initial ester content of cells.

Table 4. *Effect of pyruvate or acetate in oxygen or in nitrogen on the synthesis of* 6-O-*acetylglucose from glucose by resting cells of strain* 8508

Filter flasks (100 ml.) were charged with glucose (0·89 m-mole) in water (1 ml.), washed-cell suspension (2 ml., 22 mg. dry wt./ml., in 0·1 M-phosphate buffer, pH 7·0) and buffer (1 ml.) or sodium pyruvate or acetate (1·45 m-moles) in buffer (1 ml.). Flasks were exhausted, charged with oxygen or nitrogen, and shaken at 28° in a thermostatic bath. Samples were removed and acetylglucose was determined by quantitative paper chromatography. With this method the blank (at 0 hr.) was negligible.

	Acetylglucose formed (μmoles)			
	In oxygen		In nitrogen	
Time (hr.) ...	2·5	5	2·5	5
Glucose alone	2·1	8·3	Nil	Nil
Glucose + acetate	3·6	12·7	Nil	Nil
Glucose + pyruvate	1·1	14·2	2·3	6·4

Table 5. *Effect of sodium arsenite on the production of pyruvic acid by resting cells of strain* 8508 *in oxygen and in nitrogen*

Filter flasks (100 ml.) were charged with buffer (pH 7·0, 600 μmoles), cells (80 mg. dry wt.), glucose (890 μmoles) and 0·01 M-sodium arsenite (final concentration) in water. Water was added to give a final volume of 8·1 ml. Controls (without glucose) were set up to correct for the small amount of pyruvic acid obtained from the cells. Flasks were charged with oxygen or nitrogen in the usual way and incubated at 28° with shaking. Samples (3 ml.) were removed at 3 and 6 hr., centrifuged at 50 000 *g* and the concentration of pyruvic acid in the supernatant was determined.

	Pyruvate formed* (μmoles)			
	In oxygen		In nitrogen	
Time (hr.) ...	3	6	3	6
No inhibitor	Nil	Nil	Nil	Nil
0·01 M-Arsenite	59	140	Nil	Nil

* Corrected for endogeneous pyruvate formation (about 12 μmoles).

Experiments with cell-free extracts

We did not succeed in obtaining synthesis of the ester from glucose by cell-free extracts of strain 8508, even when sources of active acetate (acetate, pyruvate or acetylphosphate and coenzymes [coenzyme A (CoA) with cysteine or ATP, or both, with Mg^{2+} ions] were added. This failure is probably associated with the presence of a specific esterase. It was possible to show that certain enzymes concerned with acetylation were present.

6-O-*Acetyl*-D-*glucopyranose deacetylase*. The system contained extract (0·2 ml.) and acetylglucose (6·7 μmoles) in buffer (0·3 ml.). The soluble extract from alumina-ground cells of strain 8508 and *Nocardia opaca* (strain T_{16}), grown as described by Webley, Duff & Farmer (1955), had 11–15 mg. and 9–13 mg. of protein/ml. respectively. A control tube containing strain 8508 extract (boiled for 1 min.) was included. Quantitative paper chromatography showed that the amount of ester hydrolysed after incubation for 90 min. was 3·6, 0 and 0 μmoles respectively. When ATP (7 mg. as 2-amino-2-hydroxymethylpropane-1:3-diol salt and 2 mg. of $MgCl_2,6H_2O$) was added to the above system, with the strain 8508 extract there was a more rapid attack on the ester. None of the ester survived incubation for 2 hr., whereas in a control experiment without ATP or $MgCl_2,6H_2O$ about 22 % of the ester survived. Chromatograms showed that organic phosphate, probably glucose 6-phosphate, had accumulated near the origin in the 2 hr. sample (with ATP). When acetylxylose (with a secondary *O*-acetyl group; Duff, 1957) was used as a substrate in the above-mentioned system about 96 % of the ester survived incubation for 4 hr. and ATP did not affect this value. (In this case the amount of xylose produced by hydrolysis was measured as well as the initial and final concentrations of ester.)

Influence of pH *on deacetylation*. This was demonstrated with a system containing cell-free extract from alumina-ground cells of strain 8508 (0·2 ml., protein content 14–19 mg./ml.), phosphate buffer (0·1 M, pH 5, 6 and 7: 2 ml.) and acetylglucose (about 36 μmoles). The mixture was incubated at 28° and the ester content of samples (0·2 ml.) withdrawn at intervals was determined by the colorimetric method. At pH 5, 6 and 7 respectively the quantity of ester (μmoles) hydrolysed was 0·2, 0·7 and 0·7 (3 hr.) and 0·2, 0·85 and 1·3 (6 hr.).

Distribution of acetylglucose deacetylase in the cell. The strain 8508 extract (0·2 ml.), acetylglucose (about 11 μmoles in 0·1 ml. of water) and 0·1 M-phosphate buffer, pH 7·0 (0·2 ml.), were incubated at 28° in small tubes. The ester content of samples withdrawn at intervals was determined by the paper-chromatographic method. Bacteria crushed by the Hughes (1951) press gave a soluble fraction and a heavy particulate fraction (separated at 50 000 *g* for 1 hr.). Superimposed on the latter was a voluminous light particulate fraction which was separated by decantation. The particulate fractions were washed once with 0·1 M-buffer, centrifuged at 50 000 *g* in a refrigerated machine and resuspended in buffer. The dry weights of the fractions were: soluble, 33 mg./ml.; light particulate, 12 mg./ml.; heavy particulate, 20 mg./ml. (The soluble fraction had 11–13 mg. of protein/ml.) After incubation for 2 hr. with the ester as above the soluble and heavy particulate fractions had hydrolysed all the ester, but there was only 35 % hydrolysis with the light particulate fractions. After 4 hr., however, 88 % hydrolysis was recorded. When the (soluble) extract was heated at 100° for 1 min. before adding the ester there was no disappearance of the ester.

Specificity of acetylglucose deacetylase. It was possible to follow the enzymic deacetylation by a modification of the acidimetric method of Glick (1949), although the method was not sufficiently sensitive for precise work with the available extracts. Here the alumina-ground cells were extracted with 0·02 M-veronal buffer, pH 7·2. The supernatant was separated from the particulate fractions at 50 000 *g* for 1 hr. (usually 2–3 ml., containing about 10 mg. of protein/ml., was obtained) and added to acetylglucose (about 110 μmoles) in water (5 ml.). The solution was mechanically stirred in a current of carbon dioxide-free nitrogen and the pH (glass electrode) kept at 7·2–7·4 by adding 0·1 N-sodium hydroxide from a microburette. After 3–5 hr. little further acid was liberated and the pH remained practically constant.

In three experiments with these conditions strain 8508 extracts liberated acetic acid corresponding to 70–90 % of the acetylglucose contained in the solution. Extracts obtained similarly from strain 2605 and NRS 822 and 607 liberated acetic acid corresponding to 0, 0 and 17 % of acetylglucose respectively. Similar extracts from *Nocardia opaca* strain T_{16}, which is known to utilize fats (Webley, 1954), did not hydrolyse acetylglucose (two experiments). The presence of esterases in the T_{16} extract was shown by the rapid hydrolysis of diacetylfluorescein to the strongly fluorescent base (Jermyn, 1953). The strain 8508 extracts did not hydrolyse acetylcholine, acetylxylose (Duff, 1957), 6-*O*-acetylgalactose or *N*-acetylglucosamine. It was not possible to use the acidimetric method with crude commercial enzymes. Instead acetylglucose and acetylgalactose (67 μmoles in 0·5 ml. of 0·1 M-phosphate buffer, pH 7·0) were added to the enzyme (15 mg., dialysed where necessary to remove interfering sugars). The suspensions were incubated at 28° and samples withdrawn at 0, 0·25, 0·5, 1, 2, 3 and 6 hr. for paper chromatography.

Inspection or quantitative paper chromatography or both showed that lipase, pancreatin, glucose oxidase, crystalline lysozyme and Taka-diastase decomposed little or none of either ester (10% of the total amount of each ester with Taka-diastase).

Phosphotransacetylase. Table 6 shows the phosphotransacetylase activities of cell-free extracts obtained in various ways. Variable values were obtained with extracts from alumina-ground cells. The lysozyme-treated cell-free extract gave relatively high values with a marked CoA stimulation. Although the values obtained are much smaller than those given by a cell-free extract of *Clostridium kluyveri* (224 units/ml. of enzyme/hr.; Stadtman *et al.* 1951) there seems no doubt that the enzyme is present.

Acetocoenzyme A kinase. The assay was carried out on an extract of alumina-ground cells of strain 8508 as described by Jones & Lipmann (1955), the acetyl-CoA produced being trapped with hydroxylamine. As is usual with crude extracts there was a considerable production of acetohydroxamic acid without any extra CoA addition. However, the increase (about 50%) on adding CoA indicates some dependence on this cofactor. Here 0·2 ml. of the cell-free extract in 20 min. liberated 1·64 and 2·5 μmoles of acetohydroxamic acid with and without added CoA respectively. This corresponds to a minimum content of 12 units of activity/ml./hr. or a specific activity of 3·2 (units/mg. of protein in 20 min.). Another extract from cells crushed by the Hughes press gave 9·5 units of activity/ml./hr. but this was not increased with added CoA. Extracts from *N. opaca* (strain T_{16}) gave no activity.

Acylation of hydroxylamine. The system contained 0·7 M-hydroxylamine hydrochloride and 0·7 M-sodium acetate [1 ml. of each; pH was adjusted to 6·3 with 2-amino-2-hydroxymethyl-propane-1:3-diol (tris) buffer] with cell-free extract from alumina-ground bacteria (0·25 and 0·5 ml.). After incubation at 25° for 2 hr. the usual acid–ferric chloride reagent was added and the acetohydroxamic acid formed calculated from the colour density compared with a blank without enzyme. Here 14 and 9·2 μmoles of acetohydroxamic acid/ml. of enzyme respectively were found. These values may be compared with those obtained (about 2·4 μmoles of acetohydroxamic acid/ml.) by Collier & Salvonuk (1955) with an enzyme preparation from human erythrocytes.

Pyruvic oxidase. Assays for phosphate and for CoA-linked enzyme were performed as indicated by Hager & Lipmann (1955) and by Korkes (1955) respectively. No acetohydroxamic acid was obtained in either case and no oxygen uptake was recorded in the Warburg apparatus. Extracts of alumina-ground and Hughes press-crushed cells of strain 8508 were used.

DISCUSSION

Previous work on biological acetylation includes the acetylation of choline (Nachmanson & Machado, 1943; Stephenson & Rowatt, 1947), glucosamine (Chou & Soodak, 1952), carnitine (Friedman & Fraenkel, 1955), aromatic amines (Kaplan & Lipmann, 1948) and ethanol (Davies, Falkiner, Wilkinson & Peel, 1951).

The last-named system seems most closely to resemble the glucose acetylation system in that the necessary 'active acetate' is produced from the substrate in both cases.

Acetylglucose is a major, if transitory, metabolite of *B. megaterium* (NCIB 8508) growing on glucose or sucrose. No ester is formed from the other

Table 6. *Phosphotransacetylase activity in various extracts of strain* 8508

The assay was carried out according to the methods of Stadtman & Barker (1950) and Stadtman *et al.* (1951), in which the arsenolysis of acetyl-CoA (formed from acetylphosphate) is measured by the hydroxamic acid method of Lipmann & Tuttle (1945). Results have been corrected for the (small) amount of acetylphosphate decomposed in the absence of potassium arsenate. –, Signifies that no determination was made.

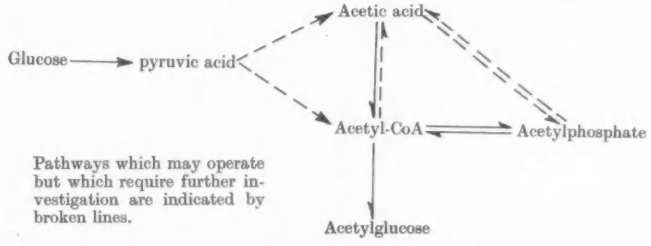
Cell-free extract	Protein content (mg./ml.)	Phosphotransacetylase (μmoles of acetylphosphate decomposed/ml. of extract/hr.)	
		No added CoA	CoA added
Cells (dried) ground with alumina	–	Nil	Nil
Cells ground with alumina	11–15	*	80
Cells ground with alumina, Dowex-treated	4–5	0·7	4·3
Cells crushed in Hughes press	9·5–13	Nil	11·4
Cells crushed in Hughes press, Dowex-treated	9·5–13	Nil	11·4
Cells lysed with lysozyme	–	44	90
Cells lysed with lysozyme, Dowex-treated	–	26	58

* Results were variable, as mentioned in the text.

carbohydrates tested or by various other bacteria grown on glucose (*B. megaterium* NCIB 7581 and NRS 697 produce acetylglucose but only about one-tenth of that produced by strain 8508). It may be that acetylglucose formation functions as a specific type of extracellular glucose-storage mechanism for the strains that produce it in an environment in which the soluble ester is retained. The ester is adsorbed more strongly on charcoal than is the parent sugar and in soil would be expected to resist leaching more than the latter. It would be difficult to establish experimentally if the strains which produce acetylglucose in liquid culture could continue to grow at its expense after the substrate (glucose) is exhausted (cf. Fig. 1) as they are spore-formers and the conditions governing spore-formation and germination in bacilli are complex (Grelet, 1957). Resting cells of strain 8508, however, readily use acetylglucose and the glucose derived from it.

Pyruvic acid would seem to be a 'key' intermediate in the synthesis of acetylglucose as (with resting cells) it accumulates in the presence of arsenite and also stimulates synthesis of the ester. Acetic acid is produced at the same time as the ester (in rather larger quantity) and the concentrations of both diminish together. Also acetic acid stimulates the synthesis of the ester at least as strongly as pyruvic acid (with resting cells, cf. Table 3).

Oxygen seems to be essential for ester synthesis from glucose but with added pyruvate and in nitrogen about 50% of the ester produced in oxygen is obtained. (Presumably the pyruvate forms 'active acetate' together with lactate or formate. A very small amount of lactate appears with resting but not with growing cells and this may be due to better aeration over a longer period with the latter.) The above evidence and the presence of coenzyme A-dependent transacetylase and acetokinase indicate the following pathways in the synthesis of acetylglucose. The final stage would require a specific 'condensing' enzyme.



Pathways which may operate but which require further investigation are indicated by broken lines.

The reactions giving a stimulation of the synthesis of acetylglucose with added acetate would presumably be mediated by acetocoenzyme A kinase, and the specific 'condensing' enzyme. Further work would be required to show whether this pathway operates normally, although the production of both acetic acid and acetylglucose as major metabolites suggests that it does.

It would be necessary to obtain an extract containing an active pyruvic oxidase system to decide whether the acetate is produced directly from pyruvate as in some bacteria (Green & Beinert, 1955), or whether the latter is metabolized directly to acetyl-CoA.

The 6-*O*-acetyl-D-glucose deacetylase in the crude extracts of strain 8508 is about evenly divided between the soluble and the particulate fractions and appears to use only acetylglucose as a substrate since 6-*O*-acetyl-D-galactopyranose and *N*-acetyl-D-glucosamine were not hydrolysed. Also various other bacterial extracts failed to hydrolyse acetylglucose, although one at least (T_{16}) had esterase activity.

The effect of pH on acetylglucose deacetylase activity and the acylation of hydroxylamine by the cell-free extract of strain 8508 recalls the behaviour of acetylcholine esterase, which mediates the acylation of choline in addition to its esterase activity (Hestrin, 1950).

The possibility that acetylglucose deacetylase is also responsible for synthesis of the ester cannot be dismissed on the basis of the evidence available.

SUMMARY

1. 6-*O*-Acetyl-D-glucopyranose is a major product of the metabolism of glucose by *Bacillus megaterium* (NCIB 8508) but not of other strains of this or various other soil organisms. The isolation of the ester is described.

2. Oxygen is essential for ester synthesis by resting cells, except when pyruvate is added. Pyruvate accumulates with arsenite-poisoned cells and the production of both acetate and ester with growing cells suggests that these acids are intermediates in ester production.

3. 6-*O*-Acetyl-D-glucopyranose deacetylase, present in cell-free extracts of the organism, did not

hydrolyse even such closely related compounds as 6-*O*-acetyl-D-galactopyranose, acetylxylose, *N*-acetylglucosamine or acetylcholine. Differential centrifuging of the crushed cells gave a soluble and two particulate fractions among which the deacetylase was approximately evenly distributed. Acetylglucose was not hydrolysed by extracts from strains of *B. megaterium* which did not produce the ester, nor by extracts from other bacteria or by commercial preparations of hydrolytic enzymes.

4. Synthesis of the ester could not be demonstrated with the cell-free extracts available, but phosphotransacetylase, acetocoenzyme A kinase and an enzyme system mediating the acetylation of hydroxylamine were present.

5. It is suggested that synthesis of the ester proceeds via pyruvic acid, acetic acid, or acetylphosphate and acetylcoenzyme A or all of these followed by a specific condensation with glucose.

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